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(57) Abstract

The present invention relates to the signalling pathways connecting DNA damage, such as that induced by ionizing radiation or alkylating agents, phosphorylation by tyrosine kinases and the c-Abl gene and gene product. More particularly, the invention involves the use of antisense molecules to selectively inhibit the expression of the c-Abl gene product following exposure of cells to DNA damaging agents, such as mitomycin C or ionizing radiation.

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DESCRIPTION

METHODS AND COMPOSITIONS INCLUDING DNA DAMAGING AGENTS AND 5 TYROSINE KINASE INHIBITORS OR ACTIVATORS

BACKGROUND OF THE INVENTION

1. Field of the Invention

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The present invention relates generally to the field of biochemical pathways. More particularly, it concerns the pathways connecting DNA damage, phosphorylation by tyrosine kinases c-abl gene and gene product.

15 2. Description of the Related Art

Current treatment methods for cancer, including radiation therapy alone, surgery and chemotherapy, are known to have limited effectiveness. Cancer mortality rates will therefore remain high well into the 21st century. The rational
20 development of new cancer treatment methods will depend on an understanding of the biology of the cancer cell at the molecular level.

Certain cancer treatment methods, including radiation therapy, involve
25 damaging the DNA of the cancer cell. The cellular response to DNA damage includes activation of DNA repair, cell cycle arrest and lethality (Hall, 1988). The signaling events responsible for the regulation of these events, however, remain unclear.

Several checkpoints in cell cycle progression control growth in response to
30 diverse positive and negative regulatory signals (Lau & Pardee, 1982). Ionizing radiation, for example, slows growth by inducing delays in G₁/S and G₂ phases of the cell cycle. The available evidence suggests that G₂ arrest is necessary for

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repair of DNA damage before entry into mitosis (Steinman *et al.*, 1991; Weinert & Hartwell, 1988). Genetic studies in *Saccharomyces cerevisiae* have demonstrated that the RAD9 protein controls G₂ arrest induced by DNA damage (Schiestl *et al.*, 1989; Murray, 1989). Mutants of the rad9 locus are unable to delay entry into mitosis following exposure to genotoxic agents and thereby replicate damaged DNA. Although the mammalian homolog of rad9 remains unidentified, other studies in various eukaryotic cells have demonstrated that entry into mitosis is regulated by a 34 kD serine/threonine protein kinase, designated p34^{cdc2} (Nurse, 1990; Pines & Hunter, 1989; Russell & Nurse, 1987).

Recent studies have shown that exposure of eukaryotic cells to ionizing radiation is associated with induction of certain early response genes that code for transcription factors. Members of the jun/fos and early growth response (EGR) gene families are activated by ionizing radiation (Sherman *et al.*, 1990; Datta *et al.*, 1992a). Expression and DNA binding of the nuclear factor kB (NF-kB) are also induced in irradiated cells (Brach *et al.*, 1991; Uckun *et al.*, 1992a). Other studies have shown that levels of the tumor suppressor p53 protein increase during X-ray-induced arrest of cells in G1 phase (Kastan *et al.*, 1991; 1992). The activation of these transcription factors presumably represents transduction of early nuclear signals to longer term changes in gene expression that constitute the response to irradiation.

Ionizing radiation also induces protein kinase C (PKC) and protein tyrosine kinase activities (Hallahan *et al.*, 1990; Uckun *et al.*, 1993). However, the specific kinases responsible for these activities and their substrates require further study. The interaction between radiation, cell signalling, phosphorylation and various other oncogenes and cellular protooncogenes has not been well studied to date.

Mitomycin C (MMC) is an antitumor antibiotic isolated from *Streptomyces caespitosus* that covalently binds to DNA (Tomasz *et al.*, 1988). This agent induces both monofunctional and bifunctional DNA lesions (Carrano *et al.*, 1979). Other studies have demonstrated that MMC stimulates the formation of hydroxyl

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radicals (Dusre *et al.*, 1989). Although the precise mechanism of action of this agent is unclear, MMC-induced cytotoxicity has been attributed to DNA alkylation and the formation of interstrand cross-links (Carrano *et al.*, 1979; Dusre *et al.*, 1989; Tomasz *et al.*, 1988). Treatment of mammalian cells with MMC is associated with inhibition of DNA synthesis and induction of sister-chromatid exchange (Carrano *et al.*, 1979). Previous work has demonstrated that MMC also enhances transcription of HIV-1 and collagenase promoter constructs transfected into HeLa cells (Stein *et al.*, 1989). These studies indicated that AP-1 is involved in MMC-induced activation of the collagenase enhancer. However, little is known about the effects of this agent on other signaling events.

Protein tyrosine phosphorylation contributes to the regulation of cell growth and differentiation. Protein tyrosine kinases can be divided into receptor-type and nonreceptor-type (Src-like) kinases (Cantley *et al.*, 1991; Hanks *et al.*, 1988; Bonni *et al.*, 1993; Larner *et al.*, 1993; Ruff-Jamison *et al.*, 1993). Several protein tyrosine kinases have been purified from the cytosolic fractions of various tissues (Nakamura *et al.*, 1988; Wong & Goldberg, 1984; Zioncheck *et al.*, 1986).

The Src-like kinases, which can associate with receptors at the plasma membrane, induce rapid tyrosine phosphorylation and/or activation of effectors such as phospholipase C- γ 1 (PLC γ 1) (Carter *et al.*, 1991), PLC γ 2 (Hempel *et al.*, 1992), mitogen-activated protein (MAP) kinase (Casillas *et al.*, 1991), GTPase activating protein (GAP) (Gold *et al.*, 1992a) and phosphatidylinositol 3-kinase (PI3-K) (Gold *et al.*, 1992b). Recent studies have demonstrated an increase in tyrosine phosphorylation following irradiation of B-lymphocyte precursors (Uckun *et al.*, 1993). Studies of p59^{fyn}, p56/p53^{lyn}, p55^{blk} and p56^{lck} activity demonstrated that these Src-family tyrosine kinases were not responsible for radiation-induced tyrosine phosphorylation (Uckun *et al.*, 1992a). These findings suggested that other protein tyrosine kinases, perhaps of the receptor-type, are involved in the response of cells to ionizing radiation.

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Varying the environmental conditions following exposure to ionizing radiation or DNA damaging agents can influence the proportion of cells that survive a given dose due to the expression or repair of potentially lethal damage (PLD). The damage is potentially lethal because while under normal circumstances it causes cell death, manipulation of the post-irradiation environment can modify the cell response. Studies show that cell survival can be increased if the cells are arrested in the cell cycle for a protracted period of time following radiation exposure, allowing repair of DNA damage. (Hall, 1988). Thus, PLD is repaired and the fraction of cells surviving a given dose of x-rays is increased if conditions are suboptimal for growth, such that cells do not have to undergo mitosis while their chromosomes are damaged.

For some diseases, e.g., cancer, ionizing radiation is useful as a therapy. Methods to enhance the effects of radiation, thereby reducing the necessary dose, would greatly benefit cancer patients. Therefore, methods and compositions were sought to enhance radiation effects by increasing the sensitivity of cells to damage from ionizing radiation and DNA damaging agents such as alkylating compounds. Cells that are irradiated or treated with DNA damaging agents halt in the cell cycle at G₂, so that an inventory of chromosome damage can be taken and repair initiated and completed before mitosis is initiated. By blocking the stress or survival response in these cells, they undergo mitosis with damaged DNA, express the mutations, and are at a greater risk of dying.

SUMMARY OF THE INVENTION

The present invention, in a general and overall sense, concerns the signalling pathways that connect DNA damage, such as that induced by ionizing radiation or alkylating agents, phosphorylation by tyrosine kinases and the c-Abl gene and gene product. More particularly, the invention involves the use of antisense molecules to selectively inhibit the expression of the c-Abl gene product following exposure of cells to DNA damaging agents, such as mitomycin C or ionizing radiation. Such an antisense molecule includes a region that is

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complementary to and capable of hybridizing with a region of the selected gene. Moreover, the antisense RNA molecules of the present invention are capable of selectively inhibiting the expression of the c-Abl gene product over that of another member of the non-receptor type of tyrosine kinases. The RNA molecule may also
5 comprise a sequence that is complementary to exon region sequences of the c-Abl gene.

In other embodiments, the invention contemplates nucleic acid molecules that comprise a coding region that expresses an antisense RNA molecule that
10 selectively inhibiting the gene product of the c-Abl gene. This DNA coding region includes an antisense RNA that is complementary to a region of the c-Abl gene. The entire nucleic acid molecule may be a DNA molecule, and this particular embodiment may encode a RNA molecule having a sequence that is complementary to the c-Abl gene sequence, or a portion thereof.

15 While it is generally believed that the antisense c-Abl gene be prepared to be complementary to an entire c-Abl gene, it is believed that shorter regions of complementary nucleic acid may be employed, so long as the antisense construct can be shown to inhibit expression of the targeted expression product. Thus, it is
20 contemplated that the nucleic acid molecule of the present invention may comprise a DNA sequence that encodes a RNA antisense molecule having a sequence that is complementary to at least a 2000 base region of the c-Abl gene. In other embodiments, the length of this RNA antisense sequence may be a 1000 base, 500 base, 100 base, or even a 10 base region of the c-Abl gene.

25 The antisense RNA of the present invention may be applied directly to cells, in the form of oligonucleotides incorporating the antisense c-Abl sequences, or nucleic acid sequences may be introduced into the cell that will encode the c-Abl sequence. It has been shown the antisense nucleotides may successfully traverse
30 cell membranes, and that such methods may be successful when liposomes are used to encapsulate the nucleic acid. Other techniques for direct insertion of the antisense construct into cells includes electroporation or calcium phosphate

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transfection. With these methods, the cells are generally removed from the host organism, treated with the constructs, and returned to the host.

The more preferred approach will involve the preparation of vectors that
5 incorporate nucleic acid sequences that encode the c-Abl sequence. It is contemplated that these vectors may either be transiently integrated into the host cell, or may be stably integrated into the host cell genome. An expression vector may comprise a gene encoding a RNA molecule complementary to the c-Abl gene and positioned under the control of a promoter, the gene positioned to effect
10 transcription of the c-Abl gene in an orientation opposite to that of vector transcription. The encoded antisense c-Abl RNA molecule is capable of selectively inhibiting the expression of the c-Abl gene product. This expression preferably occurs in a mammalian cell, and even more preferably, the mammalian cell is a human cell. Examples of suitable vectors for use within the scope of the present
15 invention include, but are not limited to, adenovirus, adeno-associated virus, retrovirus or herpes simplex virus 1.

Therefore, in certain aspects, the present invention contemplates the preparation of nucleic acid molecules that comprise a coding region that contains
20 regions complementary to and capable of hybridizing with a c-Abl gene sequence. Generally, the preferred nucleic acid molecules will be DNA sequences arranged in a vector, such as a virus or plasmid, and positioned under the control of an appropriate promoter. However, as previously set forth, the antisense RNA molecule may itself be an appropriate nucleic acid, such as retrovirus RNA into
25 which the appropriate coding sequences have been incorporated. Moreover, the nucleic acids may be introduced into cells by means of liposomes, or the like.

The particular promoter that is used within the scope of the present invention to control the expression of the antisense RNA in a vector construct is
30 not believed to be particularly crucial, as long as it is capable of expressing the antisense c-Abl DNA in the targeted cell at a rate greater than that of the gene to be inhibited. Thus, where a human cell is targeted, it will be preferred to position

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the antisense RNA coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. In a general sense, such a promoter may be of human or viral origin. The most preferred promoters are those that are capable of being expressed in a wide variety of histologic cell types, and which are capable of continuously expressing the antisense RNA. Representative
5 examples include the RSV, Herpes Simplex Virus thymidine kinase (HSV tk), the immediate early promoter from cytomegalovirus (CMV) and various retroviral promoters including LTR elements.

10 In other aspects, the invention concerns methods of selectively inhibiting the expression of a gene product of c-Abl in a cell, which includes preparing an antisense RNA molecule having a region that is complementary to and capable of hybridizing with a distinct c-Abl region, followed by introducing the antisense
15 RNA into the cell in an amount effective to inhibit the expression of the c-Abl gene product. One can test whether too much antisense c-Abl DNA has been included in an antisense construct of the present invention by simply testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences are affected. This may have importance in that it has been shown that overexpression
20 of wild type non-oncogenic c-Abl may prolong or even inhibit progression through the G1 phase (Mattioni *et al.*, 1995).

The invention also concerns methods of preparing genetic constructs for the expression of antisense c-Abl DNA, which includes incorporation of genomic DNA
25 fragments, as opposed to cDNA, into appropriate vectors for subsequent intracellular incorporation.

Also within the scope of the invention are methods of selectively inhibiting the expression of c-Abl in a cell comprising first preparing an antisense RNA
30 molecule that includes a region that is complimentary to and capable of hybridizing with a region of the c-Abl gene, followed by introducing the antisense RNA

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molecule into the cell in an amount effective to inhibit the expression of the c-Abl gene.

Also contemplated are methods of selectively inhibiting the expression of c-Abl while treating a patient with DNA damaging agents, that comprises the steps of preparing an antisense RNA molecule that includes a region that is complimentary and capable of hybridizing with a region of the c-Abl gene and administering to the patient the antisense RNA molecule in an amount effective to inhibit the expression of the c-Abl gene. Thus, a dose of a DNA damaging agent, which may be ionizing radiation, is administered to the patient in an amount effective to produce an increase in c-Abl production, which increase is abrogated by the presence of the antisense construct. The antisense RNA molecule may be introduced into the cell by introduction of a DNA molecule that encodes and is capable of expressing the antisense RNA molecule. In further embodiments, the DNA molecule is introduced into the cell by a liposome or a virus, which virus may be a retrovirus, adenovirus, or herpes simplex virus. The DNA damaging agents within the scope of the present invention include ionizing radiation and chemical agents, such as alkylating agents.

The invention also contemplates methods selectively inhibiting the expression of c-Abl in a cell, including preparing an antisense RNA molecule that comprises a sequence that is complementary to a region of the c-Abl gene and is capable of hybridizing to such a region, preparing a recombinant vector that comprises a nucleic acid sequence capable of expressing the antisense RNA in the cell, and introducing the vector into the cell in a manner that allows expression of the encoded antisense RNA at a level sufficient to inhibit gene expression.

To kill cells, such as malignant cells, using the methods and compositions of the present invention, one would generally contact a "target" cell with at least one DNA damaging agent and a c-Abl antisense molecule in a combined amount effective to kill the cell. This process may involve contacting the cells with the DNA damaging agent(s) or factor(s) and the antisense c-Abl RNA at the same

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time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the DNA damaging agent and the other composition includes the c-Abl antisense molecule.

Naturally, it is also envisioned that the target cell may be first exposed to the DNA damaging agent(s) and then contacted with a c-Abl antisense RNA molecule, or *vice versa*. In such embodiments, one would generally ensure that sufficient time elapses, so that the two agents would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both agents within about 12 hours of each other, and more preferably within about 6 hours of each other, with a delay time of only about 4 hours being most preferred. These times are readily ascertained by the skilled artisan.

The terms "contacted" and "exposed", when applied to a cell, are used herein to describe the process by which a DNA damaging agent or antisense RNA molecule are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the cell, i.e., to induce programmed cell death or apoptosis. The terms, "killing", "programmed cell death" and "apoptosis" are used interchangeably in the present text to describe a series of intracellular events that lead to target cell death.

Still further embodiments of the present invention are kits for use in killing cells, such as malignant cells, as may be formulated into therapeutic kits for use in cancer treatment. The kits of the invention will generally comprise, in suitable container means, a pharmaceutical formulation of a DNA damaging agent and a pharmaceutical formulation of a c-Abl antisense RNA molecule. These agents may be present within a single container, or these components may be provided in distinct or separate container means.

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The components of the kit are preferably provided as a liquid solution, or as a dried powder. When the components are provided in a liquid solution, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. When reagents or components are provided as a dry powder, the powder
5 can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

Although kits have been described as part of this invention, it should be noted that the use of ionizing radiation to create DNA damage is an important
10 aspect of the invention not specifically provided in kit form.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are
15 included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

20 FIG. 1A, FIG. 1B, FIG. 1C and FIG. 1D. Activation of Src-like tyrosine kinases by mitomycin C (MMC). HL-60 cells were exposed to 10^{-5} M MMC and harvested at 1 h. Cell lysates were subjected to immunoprecipitation with pre-immune rabbit serum (PIRS) (FIG. 1A); anti-Fyn antibodies (FIG. 1B); anti-Lyn
25 antibodies (FIG. 1C); and anti-Src antibodies (FIG. 1D). Phosphorylation reactions were performed in the presence of $[\gamma^{32}\text{P}]\text{ATP}$ for 10 min at 30°C . Phosphorylated protein was analyzed by 10% SDS-PAGE and autoradiography.

FIG. 2A, FIG. 2B and FIG. 2C. Activation of p56/p53^{lyn} kinase by MMC. In FIG. 2A, HL-60 cells were exposed to the indicated concentrations of MMC for
30 1 h. In FIG. 2B, cells were exposed to 10^{-5} M MMC for the indicated times. Anti-Lyn immunoprecipitates were incubated with $[\gamma^{32}\text{P}]\text{ATP}$ and enolase. Phosphorylated protein was analyzed by SDS-PAGE and autoradiography. In

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FIG. 2C, anti-Lyn immunoprecipitates were analyzed by immunoblotting with anti-Lyn.

FIG. 3A and FIG. 3B. Tyrosine phosphorylation of p56/p53^{lyn} in
5 MMC-treated cells. HL-60 cells were treated with MMC for 1 h. Cell lysates were immunoprecipitated with anti-Lyn and the immunoprecipitates were subjected to immunoblotting with anti-P-Tyr (FIG. 3A) or anti-Lyn (FIG. 3B).

FIG. 4A, FIG. 4B and FIG. 4C. MMC-induced p56/p53^{lyn} activation is
10 sensitive to tyrosine kinase inhibitors and is not a direct effect. In FIG. 4A, cells were treated with 10⁻⁵ M herbimycin A (H) or genistein (G) for 1 h and then MMC for an additional 1 h. In FIG. 4B, cells were treated with 5 x 10⁻⁵ M H7 for 1 h and then MMC for 1 h. Anti-Lyn immunoprecipitates were analyzed for phosphorylation of p56/p53^{lyn} and enolase. In FIG. 4C, cells were treated
15 MMC for 1 h. Anti-Lyn immunoprecipitates were analyzed for phosphorylation of p56/p53^{lyn} and enolase. Lysates from untreated HL-60 cells were immunoprecipitated with anti-Lyn. MMC (10⁻⁵ M) was added to the kinase reaction and incubated for 15 min. The reaction was analyzed for phosphorylation
20 of p56/p53^{lyn} and enolase.

20

FIG. 5A, FIG. 5B and FIG. 5C. Other alkylating agents active p56/p53^{lyn}.
HL-60 cells were treated with 2 x 10⁻⁶ M adozelesin (FIG. 5A), 10⁻⁵ M nitrogen
mustard (FIG. 5B) and 10⁻⁵ M cis-platinum (FIG. 5C) for 1 h. Anti-Lyn
immunoprecipitates were analyzed for phosphorylation of p56/p53^{lyn} and enolase.

25

FIG. 6A and FIG. 6B. Association of p56/p53^{lyn} and p34^{cdc2}. HL-60 cells
were treated with 10⁻⁵ M MMC for 1 h. In FIG. 6A, cell lysates were incubated
with GST or GST-Lyn proteins immobilized on beads. The resulting complexes
were separated by SDS-PAGE and analyzed by immunoblotting with anti-cdc2
30 antibody. In FIG. 6B, lysates from control (labeled HL-60) and MMC-treated cells were subjected to immunoprecipitation with anti-cdc2. The immune complexes were assayed for in vitro kinase activity by incubation with [γ -³²P]ATP. One

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aliquot of the kinase reaction was analyzed by SDS-PAGE and autoradiography. The other aliquot was washed to remove free ATP and boiled in SDS buffer to disrupt complexes. A secondary immunoprecipitation was then performed with anti-Lyn. The anti-Lyn immunoprecipitates were separated by SDS-PAGE and analyzed by autoradiography.

FIG. 7A and FIG. 7B. Effects of MMC treatment on tyrosine phosphorylation of p34^{cdc2}. HL-60 cells were exposed to MMC for 1 h. In FIG. 7A, cell lysates were subjected to immunoprecipitation with anti-cdc2. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-P-Tyr. In FIG. 7B, cell lysates were subjected to immunoprecipitation with anti-cdc2 and immunoblot analysis with anti-cdc2.

FIG. 8. Phosphorylation of cdc2 peptides by p56/p53^{lyn}. HL-60 cells were treated with MMC for 1 h. Cell lysates were subjected to immunoprecipitation with anti-Lyn. The immunoprecipitates were assayed for phosphorylation of either a cdc2 (IEKIGEGTYGVVYK; SEQ ID NO:3) or mutated cdc2 (mcdc2; Y-15 to F-15) peptide. The results represent the mean \pm S.D. of two independent studies each performed in duplicate and are normalized to control phosphorylation of the cdc2 peptide. Control cells (cross hatch); MMC-treated cells (stripes).

FIG. 9A, FIG. 9B and FIG. 9C. Activation of Src-like protein tyrosine kinases by ionizing radiation. HL-60 cells were exposed to 200 cGy ionizing radiation and harvested at 15 min or 2 hours. In FIG. 9A, Cell lysates were subjected to immunoprecipitation with anti-Fyn antibodies; in FIG. 9B, cell lysates were subjected to immunoprecipitation with anti-Lyn antibodies; and in FIG. 9C, cell lysates were subjected to immunoprecipitation with anti-Lck antibodies. Autophosphorylation reactions were performed by adding [γ -³²P]ATP for 10 min at 30°C. Phosphorylated protein was analyzed by 10% SDS-PAGE and autoradiography.

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FIG. 10A and FIG. 10B. Activation of p53/p56^{lyn} kinase by ionizing radiation. HL-60 cells were exposed to 200 cGy ionizing radiation for 5 min, 15 min, 30 min, 6 hours, 12 hours, or 24 hours. Cell lysates were subjected to immunoprecipitation with anti-Lyn. In FIG. 10A, the immunoprecipitates were analyzed in autophosphorylation reactions. In FIG. 10B, enolase phosphorylation assays are shown. Samples were separated in 10% SDS-PAGE gels and analyzed by autoradiography. The fold increase of Enolase phosphorylation, increased as measured by scintillation counting of the excised bands, is indicated at the bottom.

FIG. 11. Different doses of ionizing radiation induce activation of p53/p56^{lyn}. HL-60 cells were exposed to the indicated doses of ionizing radiation and then harvested at 12 h. Soluble proteins were subjected to immunoprecipitation with anti-Lyn. The immunoprecipitates were analyzed for phosphorylation of p56/p53^{lyn} and enolase. The fold increase in enolase phosphorylation is indicated at the bottom.

FIG. 12A and FIG. 12B. Effects of H₂O₂, NAC and protein tyrosine kinase inhibitors on activation of p56/p53^{lyn}. In FIG. 12A, HL-60 cells were either treated with H₂O₂ for the indicated times or pretreated with 30 mM NAC for 1 h, irradiated (200 cGy) and harvested at 12 h. In FIG. 12B, HL-60 cells were treated with 10 μM herbimycin (H) or 10 μM genistein (G) for 1 h, irradiated (200 cGy) and then harvested at 12 h. Cell lysates were immunoprecipitated with anti-Lyn and the immunoprecipitates were analyzed for phosphorylation of p56/p53^{lyn} and enolase.

FIG. 13A and FIG. 13B. Ionizing radiation exposure induces tyrosine phosphorylation of a 34 kD substrate. HL-60 cells were exposed to 200 cGy ionizing radiation and harvested at the indicated times. In FIG 13A, soluble proteins were subjected to immunoblot (IB) analysis with anti-P-Tyr; and in FIG. 13B soluble proteins were subjected to immunoblot (IB) analysis with anti-p34^{cdc2} antibodies. The arrow indicates the position of 34 kD signals.

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FIG. 14A and FIG. 14B. Different doses of ionizing radiation induce tyrosine phosphorylation of the 34 kD protein. HL-60 cells were exposed to the indicated doses of ionizing radiation and then harvested at 5 min. In FIG. 14A, soluble proteins were subjected to immunoblot (IB) analysis with anti-P-Tyr; and in
5 FIG. 14B, soluble proteins were subjected to immunoblot (IB) analysis with anti-p34^{cdc2} antibodies. The arrows indicate the position of the 34 kD signals.

FIG. 15A and FIG. 15B. Ionizing radiation induces tyrosine phosphorylation of p34^{cdc2}. HL-60 cells were exposed to 50 cGy ionizing
10 radiation and harvested at 5 min. Cell lysates from control and irradiated cells were subjected to immunoprecipitation (IP) with p34^{cdc2} antiserum and protein A-Sepharose. In FIG. 15A, the immunoprecipitates were subjected to immunoblot (IB) analysis with anti-P-Tyr antibodies; and in FIG. 15B, the immunoprecipitates were subjected to immunoblot (IB) analysis with anti-p34^{cdc2} antibodies.

15
FIG. 16A, FIG. 16B, FIG. 16C, FIG. 16D, FIG. 16E, and FIG. 16F. Activation of c-Abl by diverse DNA damaging agents. FIG. 16A, FIG. 16B, and FIG. 16C. U-937 or NIH3T3 cells were treated with 2 Gy ionizing radiation (IR) and harvested at 1 h. Nuclei were isolated and the nuclear proteins subjected to
20 immunoprecipitation with anti-Abl (K-12, Santa Cruz Biotechnology, San Diego, CA). *In vitro* immune complex kinase assays were performed using a GST-Crk(120-225) fusion protein as substrate (U-937, FIG. 16A, lanes 1 and 2; NIH3T3, FIG. 16B). GST-Crk(120-212) fusion protein (which lacks the critical Y221) was used as a negative control (lane 3). The anti-Abl immunoprecipitates
25 were also analyzed by immunoblotting with anti-Abl (FIG. 16C).

FIG. 16D. U-937 cells were treated with 2 Gy IR and harvested at the indicated times. Nuclear proteins were then subjected to immunoprecipitation with anti-Abl antibody. Immunoprecipitations were also performed with preimmune
30 rabbit serum (PIRS) from cells exposed to 2 Gy IR and harvested at 1 h. *In vitro* immune complex kinase assays were performed using the c-Abl substrate

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EAIYAAPFAKKK (SEQ ID NO:5). The data (percent control phosphorylation) represent the mean \pm S.E of three separate studies.

FIG. 16E and FIG. 16F. NIH3T3 cells were treated with 10 μ M CDDP for .30 min, 10 μ M MMC for 1 h or 2 Gy IR (harvested at 1 h). Nuclear proteins were subjected to immunoprecipitation with anti-Abl. Kinase assays were performed using GST-Crk(120-225) fusion protein (FIG. 16E) or EAIYAAPFAKKK (SEQ ID NO:5) peptide (FIG. 16F) as substrates.

FIG. 17A and FIG. 17B. Activation of SAP kinase activity by DNA damaging agents. FIG. 17A: NIH3T3 cells were treated with 20 Gy IR (harvested at 1 h), 10 μ M CDDP for 2 h or 10 μ M MMC for 2 h. Total lysates were immunoprecipitated with anti-SAP kinase antibody and *in vitro* immune complex kinase reactions containing GST-Jun(2-100) fusion protein were analyzed by 10% SDS-PAGE and autoradiography. FIG. 17B: Abl-/- cells were treated with 20 Gy IR (harvested at 1 h), 10 μ M CDDP for 2 h or 10 μ M MMC for 2 h. NIH3T3 cells were also treated with 10 μ M MMC for 2 h as a positive control. Total cell lysates were immunoprecipitated with anti-SAP kinase and *in vitro* immune complex kinase assays were performed using GST-Jun(2-100) as substrate.

FIG. 18A, FIG. 18B and FIG. 18C. Activation of c-Abl and SAP kinase by DNA-damaging agents in c-Abl reconstituted Abl-/- (Abl+) cells. FIG. 18A: Nuclear proteins isolated from NIH3T3, Abl-/- and Abl+ cells were subjected to immunoprecipitation with anti-Abl. The immunoprecipitates were analyzed by immunoblotting with anti-Abl. FIG. 18B: NIH3T3, Abl-/- and Abl+ cells were treated with 2 Gy IR and harvested at 1 h. Nuclei were isolated and the nuclear proteins subjected to immunoprecipitation with anti-Abl. *In vitro* immune complex kinase assays were performed using the c-Abl substrate peptide. The data (percent control phosphorylation) represent the mean \pm S.E. of two separate studies. FIG. 18C: Abl+ cells were treated with either IR (20 Gy and harvested at 1 h) or MMC (10 μ M for 2 h). Total cell lysates were immunoprecipitated with anti-SAP

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kinase antibody and *in vitro* immune complex kinase assays containing GST-Jun (2-100) fusion protein were analyzed by 10% SDS-PAGE and autoradiography.

FIG. 19A and FIG. 19B. Activation of SAP kinase by TNF is independent of c-Abl. FIG. 19A: NIH3T3 cells were treated with 2 Gy IR (harvested at 1 h) or 10 ng/ml TNF for 15 min. Nuclear lysates were immunoprecipitated with anti-Abl antibody and *in vitro* immune complex kinase assays were performed using peptide as substrate. FIG. 19B: NIH3T3 or Abl-/- cells were treated with 10 ng/ml TNF for 30 min or 20 Gy IR. Total cell lysates were immunoprecipitated with anti-SAP kinase and immune complex kinase assays were performed using GST-Jun (2-100) as substrate.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The product of the c-Abl gene is a non-receptor tyrosine kinase that is localized to the nucleus and cytoplasm. The present invention demonstrates that ionizing radiation (IR) activates c-Abl. Similar results were obtained with the alkylating agents cisplatinum and mitomycin C. The inventors also demonstrate that cells deficient in c-Abl fail to activate Jun kinase (JNK/SAP kinase) following IR or alkylating agent exposure and that reconstitution of c-Abl in these cells restores that response. In contrast, the stress response to tumor necrosis factor is stimulated by a c-Abl-independent mechanism. These findings indicate that c-Abl is involved in the stress response to DNA-damaging agents.

In addition to sharing structural features with members of the src family, c-Abl contains actin binding and DNA binding domains. The finding that c-Abl associates with the retinoblastoma (Rb) protein has suggested a potential role for c-Abl in regulating the cell cycle (Welch and Wang, 1993). Other studies have shown that overexpression of c-Abl induces an arrest in G₁ phase (Sawyers *et al.*, 1994; Mattioni *et al.*, 1995). Phosphorylation of c-Abl on multiple sites by p34^{cdc2} during mitosis has also supported a role in G₂ phase (Kipreos and Wany, 1990). The phosphorylation of c-Abl in mitotic cells inhibits DNA binding

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(Kipreos and Wang, 1992). While these findings have suggested that c-Abl contributes to regulation of the cell cycle, the present studies demonstrate that c-Abl is activated by DNA-damaging agents. DNA damage could dissociate c-Abl from a complex with other proteins and thereby contribute to interactions with potential substrates. In this context, binding of c-Abl to the first Crk SH3 domain targets phosphorylation of c-Crk on Tyr221 (Feller *et al.*, 1994; Ren *et al.*, 1994; Feller *et al.*, 1994). Since DNA damage is associated with arrest of cells in G₁ and G₂ phases, c-Abl activation could play a role in regulating these responses to genotoxic stress. Alternatively, while overexpression of c-Abl arrests cells in G₁ phase, activation of c-Abl by DNA damage may regulate distinct stress pathways that include SAP kinase.

Antisense constructs are oligo- or polynucleotides comprising complementary nucleotides to the control regions or coding segments of a DNA molecule, such as a gene or cDNA. Such constructs may include antisense versions of both the promoter and other control regions, exons, introns and exon:intron boundaries of a gene. Antisense molecules are designed to inhibit the transcription, translation or both, of a given gene or construct, such that the levels of the resultant protein product are reduced or diminished.

Nucleic acid sequences which comprise "complementary nucleotides" are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, that the larger purines will base pair with the smaller pyrimidines to form only combinations of Guanine paired with Cytosine (G:C) and Adenine paired with either Thymine (A:T), in the case of DNA, or Adenine paired with Uracil (A:U) in the case of RNA.

As used herein, the terms "complementary and/or antisense sequences" mean nucleic acid sequences that are substantially complementary over their entire length and have very few base mismatches. For example, nucleic acid sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide at thirteen or fourteen positions with only a single

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mismatch. Nucleic acid sequences which are "completely complementary" will be nucleic acid sequences which are entirely complementary throughout their entire length and have no base mismatches. In general, the longer the sequence, the larger the number of mis-matches that are tolerated.

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Antisense RNA constructs, or DNA encoding such antisense RNAs, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject. The antisense constructs have evident utility in gene inhibition embodiments. For example, U.S. Patent 4,740,463, incorporated herein by reference, describes in general methods for antagonizing the effects of an oncogene using oppositely transcribed oncogene DNA segments. Although not describing the *c-abl* gene, the methodology generally disclosed in U.S. Patent 4,740,463 may be used in connection with the DNA damaging and *c-abl* inhibition methods and compositions of the present invention.

15

PCT Patent Application WO 95/10265, incorporated herein by reference, also describes methods useful for the delivery of antisense oligos, which methods utilize a surface active non-ionic copolymer (a block copolymer). Such delivery methods may also be used in the context of the present invention. If desired, the anti-*abl* constructs may be linked to a cell-specific binding agent for enhanced delivery, as described in PCT Patent Application WO 94/23050.

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In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression (Wagner *et al.*, 1993).

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Also, U.S. Patent 5,138,045 and European Patent Application EP 431,523, each incorporated herein by reference, describe oligos having improved cellular uptake and nuclease resistance. Antisense constructs directed to the *c-abl* gene and

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modified to include nitrogenous moieties, such as polyamines and hydrazines, linked to the sugar residues, as described in U.S. Patent 5,138,045, are contemplated for use with this invention. Oligos modified according to EP 431,523 are also contemplated for use.

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Adenovirus Delivery Systems

Adenoviruses have been widely studied and well-characterized as a model system for eukaryotic gene expression. Adenoviruses are easy to grow and manipulate, and they exhibit broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, e.g., 10^9 - 10^{11} plaque-forming unit (PFU)/ml, and they are highly infective. The life cycle of Adenoviruses does not require integration into the host cell genome. The foreign genes delivered by Adenovirus vectors are expressed episomally, and therefore, have low genotoxicity to host cells. Adenoviruses appear to be linked only to relatively mild diseases, since there is no known association of human malignancies with Adenovirus infection. Moreover, no side effects have been reported in studies of vaccination with wild-type Adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

20

Adenovirus vectors have been successfully used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies demonstrated that recombinant Adenoviruses could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Successful experiments in administering recombinant Adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injection (Herz and Gerard, 1993), and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

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Generation and propagation of the current Adenovirus vectors depend on a unique helper cell line, 293, which was transformed from human embryonic kidney cells by AD5 DNA fragments and constitutively expresses E1 proteins (Graham, *et al.*, 1977). Since the E3 region is dispensable from the Adenovirus genome (Jones and Shenk, 1978), the current Adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or both regions (Graham and Prevec, 1991). In nature, Adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury, *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current Adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the Adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity.

As used herein, the term "recombinant" cell is intended to refer to a cell into which a recombinant gene, such as a gene from the adenoviral genome has been introduced. Therefore, recombinant cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced gene. Recombinant cells are thus cells having a gene or genes introduced through the hand of man. Within the present disclosure, the recombinantly introduced genes encode radiation sensitizing or radiation protecting factors and are inserted in the E1 or E3 region of the adenovirus genome. It is recognized that the present invention also encompasses genes that are inserted into other regions of the adenovirus genome, for example the E2 region.

It is understood that the adenovirus vector construct may therefore, comprise at least 10 kb or at least 20 kb or even about 30 kb of heterologous DNA and still replicate in a helper cell. By "replicate in a helper cell," it is meant that the vector encodes all the necessary *cis* elements for replication of the vector DNA, expression of the viral coat structural proteins, packaging of the replicated DNA into the viral capsid and cell lysis, and further that the *trans* elements are provided by the helper cell DNA. Replication is determined by contacting a layer of

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uninfected cells with virus particles and incubating said cells. The formation of viral plaques, or cell free areas in the cell layers is indicative of viral replication. These techniques are well known and routinely practiced in the art. It is understood that the adenoviral DNA that stably resides in the helper cell may
5 comprise a viral vector such as an Herpes Simplex virus vector, or it may comprise a plasmid or any other form of episomal DNA that is stable, non-cytotoxic and replicates in the helper cell.

By heterologous DNA is meant DNA derived from a source other than the
10 adenovirus genome which provides the backbone for the vector. This heterologous DNA may be derived from a prokaryotic or a eukaryotic source such as a bacterium, a virus, a yeast, a plant or animal. The heterologous DNA may also be derived from more than one source. For instance, a promoter may be derived from a virus and may control the expression of a structural gene from a different source
15 such as a mammal. Preferred promoters include viral promoters such as the SV40 late promoter from simian virus 40, the Baculovirus polyhedron enhancer/promoter element, RSV, Herpes Simplex Virus thymidine kinase (HSV tk), the immediate early promoter from cytomegalovirus (CMV) and various retroviral promoters including LTR elements.

20 The promoters and enhancers that comprise the heterologous DNA will be those that control the transcription of protein encoding genes in mammalian cells may be composed of multiple genetic elements. The term promoter, as used herein refers to a group of transcriptional control modules that are clustered around the
25 initiation site for RNA polymerase II. Promoters are believed to be composed of discrete functional modules, each comprising approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins. At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some
30 promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV 40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

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Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between elements is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

The heterologous DNA of the present invention may also comprise an enhancer. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities. They have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way. It is understood that any such promoter or promoter/enhancer combination may be included in the heterologous DNA of the adenoviral vector to control expression of the heterologous gene regions.

The heterologous DNA may include more than one structural gene under the control of the same or different promoters. The heterologous DNA may also include ribosome binding sites and polyadenylation sites or any necessary elements for the expression of the DNA in a eukaryotic or a mammalian cell. These vector constructs are created by methods well known and routinely practiced in the art such as restriction enzyme digestion followed by DNA ligase directed splicing of

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the various genetic elements. The heterologous DNA may further comprise a constitutive promoter. A constitutive promoter is a promoter that exhibits a basal level of activity that is not under environmental control. Some examples of constitutive promoters that may possibly be included as a part of the present invention include, but are not limited to, intermediate-early CMV enhancer/promoter, RSV enhancer-promoter, SV40 early and SV-40 late enhancer/promoter, MMSV LTR, SFFV enhancer/promoter, EBV origin of replication, or the Egr enhancer/promoter. However, it is understood that any constitutive promoter may be used in the practice of the invention and all such promoters/enhancers would fall within the spirit and scope of the claimed invention.

Another type of promoter that may comprise a portion of the heterologous DNA is a tissue specific promoter. A tissue specific promoter is a promoter that is active preferentially in a cell of a particular tissue type, such as in the liver, the muscle, endothelia and the like. Some examples of tissue specific promoters that may be used in the practice of the invention include the RSV promoter to be expressed in the liver or the surfactin promoter to be expressed in the lung, with the muscle creatine kinase enhancer combined with the human cytomegalovirus immediate early promoter being the most preferred for expression in muscle tissue, for example.

The cellular exposure to ionizing radiation is associated with transcriptional activation of certain immediate early genes that encode transcription factors (Weichselbaum *et al.*, 1991). These genes include members of the jun-fos, NF-kB and early growth response (EGR-1) gene families (Hallahan *et al.*, 1992; Datta *et al.*, 1992; Brach *et al.*, 1991). The induction of these genes following x-irradiation may represent cellular responses to oxidative stress (Datta *et al.*, 1992; Brach *et al.*, 1991; Datta *et al.*, 1993). Previous studies have demonstrated that induction of Egr-1 gene transcription is mediated by activation of CC(A+T rich)₆GG(CArG) motifs in the Egr-1 promoter (Datta *et al.*, 1993; Datta *et al.*, 1992).

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In certain embodiments, the present invention is a method of expressing an antisense RNA molecule in a mammalian cell. This method would comprise the steps of obtaining an adenoviral vector construct comprising more than 7.5 kb of heterologous DNA, replicating the adenoviral vector construct in a helper cell, obtaining virion particles produced by the helper cells and infecting mammalian cells with the virion particles.

The c-Abl antisense RNA molecule to be expressed as described in the preceding paragraph may of any origin, for example, an animal or a human gene. Preferably the adenovirus vector construct contains a deletion in the E1 or E3 region of the genome and the foreign gene is inserted in its place.

The virion plaques that would be produced by the replicating viral vector and would thus lyse the host cell can be obtained by any acceptable means. Such means would include filtration, centrifugation or preferably physical touching of viral plaques. All such methods of obtaining virion particles and infecting mammalian cells with the particles are well known to those of skill in the art.

The examples of preferred embodiments disclosed herein utilize human adenovirus type 5. Type 5 virus was selected because a great deal of biochemical and genetic information about the virus is known, and it has historically been used for most constructions employing adenovirus as a vector. It is understood, however, the adenovirus may be of any of the 42 different known serotypes of subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the method of the present invention.

By employing a promoter with well-known properties, the level and pattern of expression of c-Abl antisense RNA following infection can be optimized. For example, selection of a promoter which is active specifically in certain cell types will permit tissue-specific expression of the antisense molecule.

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In still further embodiments, the invention relates to a method for increasing c-Abl antisense RNA levels in a subject comprising administering to the subject an effective amount of a pharmaceutical composition which includes the adenovirus vector/c-Abl antisense RNA construct. The inventors propose that an effective
5 amount of the vector construct will involve the administration of from about 10^8 to 10^{11} virus particles, which may be given either as a single bolus injection directly into the tumor or as a systemic intravenous infusion over several hours.

Other than the requirement that the adenovirus vector be replication
10 defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-
15 defective adenovirus vector for use in the method of the present invention. This is because Adenovirus type 5 is a human adenovirus of which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

In further embodiments, the invention relates to pharmaceutical
20 compositions wherein the adenovirus vector/c-Abl antisense RNA gene construct is dispersed in a pharmacologically acceptable solution or buffer. Preferred solutions include neutral saline solutions buffered with phosphate, lactate, or Tris, containing sucrose or glycerol, and the like. Of course, one will desire to purify the vector
25 sufficiently to render it essentially free of undesirable contaminant, such as defective interfering adenovirus particles or endotoxins and other pyrogens such that it will not cause any untoward reactions in the individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

30 In that adenovirus is a virus that infects humans, there may be certain individuals that have developed antibodies to certain adenovirus proteins. In these circumstances, it is possible that such individuals might develop an immunological

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reaction to the virus. Thus, where an immunological reaction is believed to be a possibility, one may desire to first test the subject to determine the existence of antibodies. Such a test could be performed in a variety of accepted manners, for example, through a simple skin test or through a test of the circulating blood levels of adenovirus-neutralizing antibodies. In fact, under such circumstances, one may desire to introduce a test dose of on the order of 1×10^5 to 1×10^6 or so virus particles. Then, if no untoward reaction is seen, the dose may be elevated over a period of time until the desired dosage is reached, such as through the administration of incremental dosages of approximately an order of magnitude.

The particular cell line used to propagate the recombinant adenoviruses of the present invention is not critical to the present invention. The recombinant adenovirus vectors can be propagated on, *e.g.*, human 293 cells, or in other cell lines that are permissive for conditional replication-defective adenovirus infection, *e.g.*, those which express adenovirus E1A gene products "in trans" so as to complement the defect in a conditional replication-defective vector. Further, the cells can be propagated either on plastic dishes or in suspension culture, in order to obtain virus stocks thereof.

Liposome Delivery Systems

Liposomes have been used for more than a decade to introduce exogenous DNA into cells (Mukherjee *et al.*, 1978; Nicolau *et al.*, 1983). The term liposome is used to describe different forms of surfactant vesicles consisting of one or more concentric lipid bilayer spheroids surrounding an aqueous space. Classical liposomes consist of fatty acid esters and fat-alcohol ethers of glycerol phosphatides. Their net charge is negative under physiological pH conditions due to phosphate groups. In recent years, liposomes bearing a positive charge derived from quaternary ammonium groups such as *N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*-trimethylammonium chloride (DOTMA)² (Felgner *et al.*, 1987) have been introduced. These cationic liposomes interact strongly with cellular membrane which are themselves negatively charged. In contrast to classic liposomes they do

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not encapsulate or entrap DNA but bind it at their surface. Another group of liposomes consists of nonionic surfactant vesicles. While classical phospholipid-based liposomes are of low toxicity, the toxicity and antigenicity of the partially synthetic cationic and nonionic liposomes have not been rigorously evaluated.

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As used herein, exemplary liposome preparations include, but are not limited to DOTMA, 1,2-dioleoyloxypropyl-3-trimethyl ammonium bromide; DOPE, dioleoylphosphatidylethanolamine; POPE, palmitoyloleoylphosphatidylethanolamine; DMPE, dimyristoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; 10 DSPE, distearoylphosphatidylethanolamine; PMME, dioleoylphosphatidylmonomethylethanolamine; PDME, dioleoylphosphatidyl dimethylethanolamine; DOPC, dioleoylphosphatidylcholine; CPE, dioleoylphosphatidylcaprylamine; DPE, dioleoylphosphatidyl dodecylamine; 15 DORI, 1,2-dioleoyl-3-dimethyl-hydroxyethyl ammonium bromide (Dioleoyl Rosenthal Inhibitor); or DORIE, 1,2-dioleoyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide;

Herpes Simplex Virus

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The present invention also embodies a method using HSV-1 for delivering genes for gene therapy. In an exemplary embodiment, the method involves combining the gene used for gene therapy with the HSV-1 virus rendered non-pathogenic. The gene and the virus are then combined with a pharmacologically acceptable carrier in order to form a pharmaceutical composition. This 25 pharmaceutical composition is then administered in such a way that the mutated virus containing the gene for therapy, or the HSV-1 wild type virus containing the gene, can be incorporated into cells at an appropriate area. The use of the HSV-1 virus with a specific mutation in the $\gamma_1 34.5$ gene provides a method of therapeutic treatment of tumorigenic diseases both in the CNS and in all other parts of the 30 body (Chou 1992). The " $\gamma_1 34.5$ minus" virus can induce apoptosis and thereby cause the death of the host cell, but this virus cannot replicate and spread (Chou

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1992). Therefore, given the ability to target tumors within the CNS, the $\gamma_{134.5}$ minus virus has proven a powerful therapeutic agent for hitherto virtually untreatable forms of CNS cancer. Furthermore, use of substances, other than a virus, which inhibit or block expression of genes with anti-apoptotic effects in target tumor cells can also serve as a significant development in tumor therapy and in the treatment of herpes virus infection, as well as treatment of infection by other viruses whose neurovirulence is dependent upon an interference with the host cells' programmed cell death mechanisms. The procedures to generate the above recombinant viruses are those published by Post and Roizman (1981), and U.S. Patent No. 4,769,331, incorporated herein by reference.

Retroviral Delivery

Retroviruses may also be used to deliver the antisense RNA constructs to the host target tissues. These viruses in which the 3' LTR (linear transfer region) has been inactivated. They are enhancerless 3'LTR's, often referred to as self-inactivating viruses because after productive infection into the host cell, the 3'LTR is transferred to the 5' end and both viral LTR's are inactive with respect to transcriptional activity. A use of these viruses well known to those skilled in the art is to clone genes for which the regulatory elements of the cloned gene are inserted in the space between the two LTR's. An advantage of a viral infection system is that it allows for a very high level of infection into the appropriate recipient cell.

Pharmaceutical Compositions

In another aspect, the present invention contemplates a pharmaceutical composition comprising a therapeutically effective amount of at least one genetic construct of the present invention and a physiologically acceptable carrier.

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A therapeutically effective amount of a genetic construct that is combined with a carrier to produce a single dosage form varies depending upon the host treated and the particular mode of administration.

5 As is well known in the art, a specific dose level for any particular patient depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination, and the severity of the particular disease undergoing therapy.

10

A composition of the present invention is typically administered orally or parenterally in dosage unit formulations containing standard, well known nontoxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes subcutaneous injections, intravenous, 15 intramuscular, intraarterial injection, or infusion techniques.

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions are formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also 20 be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, 25 fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

30 A genetic construct of the present invention can also be complexed with a poly(L-Lysine)(PLL)-protein conjugate such as a transferrin-PLL conjugate or an asialoorosomucoid-PLL conjugate.

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Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, syrups, solutions, suspensions, and elixirs containing inert diluents commonly used in the art, such as water. Such compositions can also comprise adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I

Alkylating Agents Activate the Lyn Tyrosine Kinase and Promote Tyrosine Phosphorylation of p34^{cdc2}

A. Materials and Methods

Cell culture. HL-60 cells were grown in RPMI-1640 medium containing 15% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 mM non-essential amino acids. Cells were treated with MMC (Sigma Chemical Co., St. Louis, MO), adozelesin (Sigma), cis-platinum (Sigma), nitrogen mustard (Sigma), genistein (GIBCO/BRL, Gaithersburg, MD), herbimycin A (GIBCO/BRL) and H-7 (Seikagaku America Inc., Rockville, MD). Cell viability was determined by trypan blue exclusion.

Immune complex kinase assays. Cells ($2-3 \times 10^7$) were washed twice with ice cold phosphate buffered saline (PBS) and lysed in 2 ml of lysis buffer (20

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mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT and 10 mg/ml of leupeptin and aprotinin). After incubation on ice for 30 min, insoluble material was removed by centrifugation at 14000 rpm for 10 min at 4°C. Soluble proteins were precleared
5 by incubating with 5 mg/ml rabbit-anti-mouse IgG for 1 h at 4°C and then for an additional 30 min after addition of protein A-sepharose.

The supernatant fraction was incubated with pre-immune rabbit serum, anti-Fyn, anti-Lyn, anti-Src (UBI, Lake Placid, NY) or anti-cdc2 (sc-54, Santa Cruz
10 Biotechnology, Santa Cruz, CA) antibodies for 1 h at 4°C followed by 30 min after addition of protein A-sepharose. The immune complexes were washed three times with lysis buffer and once with kinase buffer (20 mM HEPES, pH 7.0, 10 mM MnCl₂ and 10 mM MgCl₂) and resuspended in 30 ml of kinase buffer containing 1 mCi/ml [γ -³²P]ATP (3000 Ci/mmol; NEN, Boston, MA) with and without 5-8 mg
15 of acid-treated enolase (Sigma). The reaction was incubated for 15 min at 30°C and terminated by the addition of 2x SDS sample buffer. The proteins were separated in 10% SDS-polyacrylamide gels and analyzed by autoradiography. Radioactive bands were excised from certain gels and quantitated by scintillation
20 counting.

Immune complexes were also resuspended in 30 ml kinase buffer containing 1 mCi/ml [γ -³²P]ATP and either 100 mM cdc2 peptide (amino acids 7 to 20; IEKIGEGTYGVVYK; SEQ ID NO:3) or 100 mM mutated cdc2 peptide with Phe-15 substituted for Tyr-15 (IEKIGEGTEGVVYK; SEQ ID NO:4). The
25 reactions were incubated for 15 min at 30°C and terminated by spotting on P81 phosphocellulose discs (GIBCO/BRL). The discs were washed twice with 1% phosphoric acid and twice with water before analysis by liquid scintillation counting.

30 **Immunoblot analysis.** Immune complexes bound to protein A-sepharose were prepared as for the autophosphorylation assays. Proteins were separated in 10% SDS-polyacrylamide gels and transferred to nitrocellulose paper. The residual

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binding sites were blocked by incubating the filters in 5% dry milk in PBST (PBS/0.05% Tween-20) for 1 h at room temperature. The blots were subsequently incubated with anti-cdc2 or anti-phosphotyrosine (anti-P-Tyr; Mab 4G10, UBI). After washing twice with PBST, the filters were incubated for 1 h at room temperature with anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma) in 5% milk/PBST. The filters were then washed and the antigen-antibody complexes visualized by the ECL detection system (Amersham, Arlington Heights, IL).

Coimmunoprecipitation. Immunoprecipitations were performed with anti-p34^{cdc2} at 5 mg/ml cell lysate. Immune complexes were collected on protein A-Sepharose beads (Pharmacia), washed three times with lysis buffer and twice with kinase buffer, resuspended in kinase buffer and then incubated for 10 min at 30°C in the presence of 1 mCi/ml [γ -³²P]ATP. One aliquot of the kinase reaction was subjected to SDS-PAGE and autoradiography. The other aliquot was washed in lysis buffer to remove free ATP and then boiled in 20 mM Tris-HCl, pH 8.0 containing 0.5% SDS and 1 mM DTT to disrupt protein-protein interaction. After dilution to 0.1% SDS, a secondary immunoprecipitation was then performed by adding anti-Lyn antibody and protein A-Sepharose beads. The anti-Lyn immunoprecipitates were then subjected to SDS-PAGE and autoradiography.

Fusion protein binding assays. The plasmid encoding a glutathione S-transferase (GST)-Lyn (amino acids 1 to 243) fusion protein was obtained from T. Pawson, Toronto, Canada and transfected into *E. coli* DH5a (Pleiman *et al.*, 1993). The fusion protein was induced with IPTG, purified by affinity chromatography using glutathione-Sepharose beads (Pharmacia) and equilibrated in lysis buffer. HL-60 cell lysates were incubated with 50 mg immobilized GST or GST-Lyn for 2 h at 4°C. The protein complexes were washed three times with lysis buffer and boiled for 5 min in SDS sample buffer. The complexes were then separated in 10% SDS-PAGE and subjected to silver staining or immunoblot analysis with anti-cdc2.

B. Results

Previous studies have demonstrated that HL-60 cells express the p59^{fyn}, p56/p53^{lyn} and pp60^{c-src} tyrosine kinases (Barnekow & Gessler, 1986; Gee *et al.*, 1986; Katagiri *et al.*, 1991). In this example, the inventors have shown that certain of these tyrosine kinases are activated during treatment of HL-60 cells with MMC.

Immunoprecipitates from control and MMC-treated cells were assayed for autophosphorylation. There was no detectable kinase activity in precipitates obtained with pre-immune rabbit serum (FIG. 1A). Other studies with an anti-Fyn antibody demonstrated that autophosphorylation of p59^{fyn} is decreased at 1 h of MMC treatment (FIG. 1B). Similar results were obtained at multiple time points through 6 h of MMC exposure. In contrast, immunoprecipitates with anti-Lyn demonstrated an increase in p56/p53^{lyn} activity as a result of MMC exposure (FIG. 1C). The finding that anti-Src immunoprecipitates also exhibited a decrease in pp60^{c-src} activity in MMC-treated cells (FIG. 1D) suggests that MMC exposure is associated with selective activation of p56/p53^{lyn}.

Activation of p56/p53^{lyn} was confirmed at different concentrations of MMC and by assaying for phosphorylation of the substrate protein enolase. Increases in p56/p53^{lyn} activity were found at 10⁻⁸ and 10⁻⁷ M MMC, while more pronounced stimulation of this kinase was apparent at 10⁻⁶ and 10⁻⁵ M (FIG. 2A). The results further demonstrate that p56/p53^{lyn} activity is rapidly induced in MMC-treated cells. Increases in MMC-induced phosphorylation of p56/p53^{lyn} and enolase were first detectable at 30 min (4.2-fold increase for enolase) and persisted through at least 12 h (4.1-fold for enolase) of drug exposure (FIG. 2B). The induction of p56/p53^{lyn} activity was not related to cell death since viability as determined by trypan blue exclusion was >90% at 12 h of MMC treatment.

Immunoblot analysis was also performed to determine whether the increases in p56/p53^{lyn} activity were due to a greater abundance in protein. The results demonstrate similar levels of p56/p53^{lyn} protein (FIG. 2C). These findings

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supported a rapid and prolonged activation of p56/p53^{lyn} in response to MMC treatment.

In order to confirm that activation of p56/p53^{lyn} is associated with tyrosine phosphorylation, the anti-Lyn immune complexes were assayed by immunoblotting with anti-P-Tyr. The results demonstrate an increase in tyrosine phosphorylation of p56/p53^{lyn} from MMC-treated as compared to control cells (FIG. 3A). Analysis of the anti-Lyn immunoprecipitates by immunoblotting with anti-Lyn confirmed the presence of similar levels of protein after MMC treatment (FIG. 3B).

The involvement of tyrosine phosphorylation was further supported by the demonstration that pretreatment of cells with the tyrosine kinase inhibitors, genistein (Akiyama *et al.*, 1987) and herbimycin A (Uehara *et al.*, 1989) completely blocks the stimulation of p56/p53^{lyn} activity associated with MMC treatment (FIG. 4A). In contrast, pretreatment with the isoquinoline sulfonamide inhibitor of serine/threonine protein kinases, H-7 (Hidaka *et al.*, 1984), had no detectable effect on the MMC-induced activity (FIG. 4B). These effects of MMC on induction of p56/p53^{lyn} could be related to direct interaction of this agent with Lyn kinase. However, incubation of anti-Lyn immune complexes in the presence of MMC was associated with a decrease in kinase activity (FIG. 4C). Taken together, these findings indicated that MMC induces the tyrosine kinase activity of p56/p53^{lyn} by an indirect mechanism.

The available evidence indicates that MMC acts as a monofunctional and bifunctional alkylating agent (Carrano *et al.*, 1979). Consequently, adozelesin, another monofunctional but structurally distinct alkylating agent (Bhuyan *et al.*, 1992; Hurley *et al.*, 1984), was investigated. The results demonstrate that treatment of HL-60 cells with adozelesin is similarly associated with stimulation of p56/p53^{lyn} and enolase phosphorylation (FIG. 5A). Other studies were performed with agents that also induce the formation of DNA cross-links. Nitrogen mustard, an agent that forms monoadducts and DNA interstrand cross-links (Ewig & Khon, 1977; Hartley *et al.*, 1992), was effective in inducing p56/p53^{lyn} activity

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(FIG. 5B). Moreover, treatment of cells with cis-platinum, an agent that forms intrastrand cross-links (Sherman & Lippard, 1987), was associated with stimulation of the p56/p53^{lyn} kinase (FIG. 5C). These findings indicated that the response of cells to diverse alkylating-type agents induces activation of p56/p53^{lyn}.

In order to examine the significance of p56/p53^{lyn} activation, the association of this kinase with specific intracellular proteins that undergo tyrosine phosphorylation in MMC-treated cells was investigated. This issue was initially addressed using a GST-Lyn fusion protein to identify molecules which interact with p56/p53^{lyn}. Lysates from MMC-treated cells were incubated with immobilized GST or GST-Lyn. Analysis of the adsorbates by SDS-PAGE and staining demonstrated the presence of a 34 kD protein.

The inventors assayed the adsorbates for reactivity with anti-cdc2. The results indicate that p34^{cdc2} associates with the GST-Lyn fusion protein and not the GST control (FIG. 6A). The potential interaction between p56/p53^{lyn} and p34^{cdc2} was further examined in coimmunoprecipitation studies. Lysates of control and MMC-treated cells were subjected to immunoprecipitation with anti-cdc2 and the immunoprecipitates were assayed for autophosphorylation (FIG. 6B). One aliquot of the in vitro kinase reaction was assayed by SDS-PAGE and autoradiography. While immunoprecipitates from MMC-treated cells exhibited phosphorylation of 53-56 kD proteins, there was little if any of this activity in control cells (FIG. 6B). In order to determine whether the anti-cdc2 immunoprecipitates contain p56/p53^{lyn}, the other aliquot of the in vitro kinase reaction was treated to disrupt protein complexes and then subjected to immunoprecipitation with anti-Lyn. The results demonstrate increased levels of autophosphorylated p56/p53^{lyn} when assaying MMC-treated as compared to control cells (FIG. 6B).

The finding that MMC exposure induces an interaction between p56/p53^{lyn} and p34^{cdc2} prompted further studies to determine whether p34^{cdc2} exhibits increased tyrosine phosphorylation in MMC-treated cells. Immunoprecipitation of p34^{cdc2} and then immunoblotting of the precipitates with anti-P-Tyr demonstrated

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an increase in reactivity as a result of MMC treatment (FIG. 7A). Reprobing the filter with the anti-cdc2 antibody demonstrated similar levels of p34^{cdc2} protein (FIG. 7B). Since these findings indicated that MMC treatment is associated with increased tyrosine phosphorylation of p34^{cdc2}, other studies were performed to determine whether p56/p53^{lyn} can phosphorylate p34^{cdc2} in vitro.

In order to study a potential phosphorylation site for Src-like kinases located at Tyr-15 of p34^{cdc2}, synthetic peptides were prepared with sequences derived from amino acids 7 to 20 of p34^{cdc2} and another with substitution at Tyr-15 with Phe-15. While anti-Lyn immune complexes from control cells phosphorylated the cdc2 peptide, similar complexes from MMC-treated cells exhibited nearly a 2-fold stimulation in this activity (FIG. 8). In contrast, there was little phosphorylation of the mutated cdc2 peptide with anti-Lyn complexes from control or MMC-treated cells (FIG. 8). These findings indicated that p56/p53^{lyn} phosphorylates the Tyr-15 site of p34^{cdc2}.

The present results demonstrate that treatment of HL-60 cells with MMC is associated with selective activation of the p56/p53^{lyn} tyrosine kinase. These findings are not limited to HL-60 cells since other cell lines, for example U-937 myeloid leukemia cells, also respond to this agent with increases in p56/p53^{lyn} activity.

The lyn gene encodes two forms of the tyrosine kinase, p56^{lyn} and p53^{lyn}, due to alternate mRNA splicing (Yamanashi *et al.*, 1987; Yamanashi *et al.*, 1989). As a member of the Src-like family of tyrosine kinases, p56/p53^{lyn} is related to pp60^{c-src} and p59^{fyn} (Cantley *et al.*, 1991). However, only p56/p53^{lyn} was activated in MMC-treated cells. These kinases are often associated with cell surface receptors at the interface between the cell membrane and cytoplasm. Studies of p56/p53^{lyn} in B cells have demonstrated an association with the B-cell antigen receptor (Pleiman *et al.*, 1993; Yamanashi *et al.*, 1992). Engagement of the B-cell antigen receptor induces activation of p56/p53^{lyn}, as well as other Src-like kinases, and tyrosine phosphorylation of substrates that include PLC γ 2,

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MAP kinase and GAP (Pleiman *et al.*, 1993). Other studies have shown that p56/p53^{lyn} associates with the 85 kDa α -subunit of PI 3-K and induces PI 3-K activity (Yamanashi *et al.*, 1992). Thus, p56/p53^{lyn} is capable of associating with and phosphorylating diverse downstream effector molecules.

5 Although the cellular effects of alkylating agents such as MMC are generally attributed to DNA damage, their action may be related to alkylation of RNA or protein. The demonstration that MMC treatment of intact cells is associated with activation of p56/p53^{lyn} raised the possibility that this effect might be due to direct alteration of Lyn protein. p56/p53^{lyn} activity was however
10 decreased in vitro by incubation of anti-Lyn immune complexes with MMC. In order to address the possibility that MMC-induced activation of p56/p53^{lyn} is related to formation of DNA lesions, another agent, adozelesin, was used that covalently binds to the N-3 of adenine within the minor groove of DNA (Bhuyan
15 *et al.*, 1992; Hurley *et al.*, 1984). Adozelesin also induces p56/p53^{lyn} activity.

HL-60 cells also respond similarly to other alkylating agents, such as nitrogen mustard which reacts predominantly with guanines by alkylation of their N-7 positions or forms DNA interstrand cross-links (Ewig & Khon, 1977; Hartley
20 *et al.*, 1992). Moreover, p56/p53^{lyn} activity was stimulated by cis-platinum which induces intrastrand cross-links (Sherman & Lippard, 1987). Thus, structurally distinct agents that damage DNA by diverse mechanisms are capable of inducing p56/p53^{lyn} activity. Recent studies have demonstrated that treatment of HeLa cells with ultraviolet (UV) irradiation is associated with increases in the catalytic activity
25 of c-Src and c-Fyn, but not that of c-Yes (Devary *et al.*, 1992). Taken together with the absence of detectable pp60^{c-src} or p59^{fyn} activation in MMC-treated HL-60 cells, these results suggest that induction of these tyrosine kinases may be cell-type or agent specific.

30 The p34^{cdc2} serine/threonine protein kinase controls entry of cells into mitosis (Nurse, 1990; Pines & Hunter, 1990). This kinase is regulated by networks of kinases and phosphatases that appear to respond to the state of DNA replication.

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Activation of p34^{cdc2} involves association with cyclin B and posttranslational modifications of the p34^{cdc2}/cyclin B complex (Norbury & Nurse, 1992). Phosphorylation of p34^{cdc2} on Thr-161 is required for activation (Atherton-Fessler *et al.*, 1993; Desai *et al.*, 1992; Solomon *et al.*, 1992), while Tyr-15
5 phosphorylation results in inhibition of both p34^{cdc2} activity and entry of cells into mitosis (Gould & Nurse, 1989; Gould *et al.*, 1990).

Studies have demonstrated that treatment of mammalian cells with alkylating and other DNA-damaging agents is associated with G₂ arrest (Konopa,
10 1988; Lau & Pardee, 1982; Tobey, 1975). However, the precise mechanisms responsible for this effect have remained unclear. Exposure of cells to ionizing radiation is associated with rapid inhibition of p34^{cdc2} activity and G₂ arrest (Lock & Ross, 1990). Other studies have demonstrated that arrest of nitrogen
mustard-treated cells at G₂ is temporally related to formation of DNA cross-links
15 and p34^{cdc2} inhibition (O'Connor *et al.*, 1992). In the present studies, it is demonstrated that MMC treatment results in rapid tyrosine phosphorylation of p34^{cdc2}. Similar findings have been obtained in cells treated with ionizing
radiation (see following Examples). This modification of p34^{cdc2} is associated
with loss of kinase activity as determined by assaying anti-cdc2 immunoprecipitates
20 for phosphorylation of H1 histone. Thus, the phosphorylation of p34^{cdc2} on tyrosine appears to represent in part the response of mammalian cells to DNA
damage and may contribute to G₂ arrest by inhibition of p34^{cdc2} activity.

The available evidence indicates that the p107^{wee1} dual-specificity kinase is
25 responsible for phosphorylation of p34^{cdc2} on Tyr-15 (Featherstone & Russell, 1991; Parker *et al.*, 1991; Parker *et al.*, 1992). While p107^{wee1} appears to control p34^{cdc2} activity to ensure completion of S-phase, other studies suggest that p107^{wee1} is not required for the DNA-damage-dependent mitotic checkpoint. In this
context, normal mitotic arrest has been observed after irradiation of
30 *Schizosaccharomyces pombe* cells with a defective or missing *wee1* gene (Barbet & Carr, 1993). Other studies have shown that p34^{cdc2} is phosphorylated on tyrosine in yeast *wee1* minus mutants (Gould *et al.*, 1990). The present results in

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mammalian cells suggest that regulation of p34^{cdc2} following exposure to alkylating agents involves activation of p56/p53^{lyn}. The association of p56/p53^{lyn} and p34^{cdc2} in MMC-treated cells, as well as the finding that p56/p53^{lyn} can phosphorylate the Tyr-15 site of p34^{cdc2} in vitro, support the possibility that

5 p56/p53^{lyn} contributes to signaling from the mitotic checkpoint that monitors for alkylating agent-induced damage.

EXAMPLE II

Ionizing Radiation Activates the Lyn Tyrosine Kinase and Promotes Tyrosine Phosphorylation of p34^{cdc2}

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Treatment of human HL-60 myeloid leukemia cells with ionizing radiation is associated with activation of the Lyn tyrosine kinase. The lyn gene encodes two forms of this kinase, p56^{lyn} and p53^{lyn}, as a result of alternate splicing (Yamanashi

15 *et al.*, 1987; 1989). Both p56/p53^{lyn}, but not certain other Src-related kinases, are activated in irradiated HL-60 cells. Activation of p56/p53^{lyn} represents a signaling pathway distinct from those involved in X-ray-induced early response gene expression.

20

HL-60 myeloid leukemia cells were grown in RPMI-1640 medium containing 15% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 1mM non-essential amino acids. Cells in logarithmic growth phase were suspended in complete RPMI-1640 medium with 0.5% FBS 18 hours prior to

25 irradiation.

30

Irradiation was performed at room temperature using a Gammacell 1000 (Atomic Energy of Canada, Ottawa) under aerobic conditions with a ¹³⁷Cs source emitting at a fixed dose rate of 13.3 Gy/min as determined by dosimetry. HL-60 cells were also treated with 50 mM H₂O₂ (Sigma Chemical Co., St. Louis, MO), 30 mM N-acetyl cysteine (NAC; Sigma), 10 μM genistein (GIBCO/BRL, Gaithersburg, MD) or 10 μM herbimycin A (GIBCO/BRL).

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Cells ($2-3 \times 10^7$) were washed twice with ice cold phosphate buffered saline (PBS) and lysed in 2 ml of lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 10 mg/ml of leupeptin and aprotinin). After incubation on ice for 30 min, insoluble material was removed by centrifugation at 1400 rpm for 10 min at 4°C. Soluble proteins were precleared by incubation with 5mg/ml rabbit anti-mouse IgG for 1 hour at 4°C and then addition of protein A sepharose for 30 min.

The supernatants were incubated with 2.5 μ l of anti-human Fyn, 2 μ l of anti-human Lyn, 3 μ l of anti-human Lck (N-terminal) or 3 μ l of anti-Src antibody (UBI, Lake Placid, NY) for 1 hour at 4°C followed by 30 min with protein A-sepharose. The immune complexes were washed three times with lysis buffer, once with kinase buffer (20 mM HEPES, pH 7.0, 10 mM $MnCl_2$ and 10 mM $MgCl_2$) and resuspended in 30 μ l of kinase buffer containing 1 mCi/ml [γ - ^{32}P]ATP (3000 Ci/mmol; NEN, Boston, MA). The reaction was incubated for 10 min at 30°C and terminated by the addition of 2x SDS sample buffer. The proteins were resolved in 10% SDS-polyacrylamide gels, dried and analyzed by autoradiography.

Immune complexes as prepared for autophosphorylation assays were washed three times with lysis buffer and once with kinase buffer. The beads were resuspended in 30 μ l of kinase buffer containing 1 mCi/ml [γ - ^{32}P]ATP and 3-5 mg of acid treated enolase (Sigma). The reaction was incubated for 10 min at 30°C and terminated by the addition of 2 x SDS sample buffer. The proteins were resolved by 10% SDS-PAGE. Equal loading of the enolase was determined by staining with Coomassie blue. The gels were then destained and analyzed by autoradiography. Radioactive bands were also excised from the gel and quantitated by scintillation counting.

Previous studies have demonstrated that p59^{fyn} and p56/p53^{lyn} are expressed in HL-60 cells (Katagiri *et al.*, 1991). Using autophosphorylation assays, the present inventors herein show that irradiation of HL-60 cells with 200 cGy was associated with little if any change in p59^{fyn} activity at 15 min and 12 hours

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(FIG. 9A). A more detailed analysis between those time points revealed similar findings. In contrast, p56/p53^{lyn} activity was increased at both 15 min and 12 hours after irradiation as compared to that in untreated cells (FIG. 9B). Studies of p56^{lck} demonstrated little detectable activity in HL-60 cells before or after exposure to ionizing radiation (FIG. 9C). These findings show that p56/p53^{lyn} is selectively activated in HL-60 cells by ionizing radiation. This conclusion is further supported by the absence of an increase in c-Src activity following irradiation.

HL-60 cells were also irradiated with 200 cGy and immunoprecipitates assayed for both p56/p53^{lyn} autophosphorylation and enolase (a substrate protein) phosphorylation. Irradiation was associated with an increase in p56/p53^{lyn} autophosphorylation at 5 min that persisted through 12 hours (FIG. 10A). However, assays at 24 hours after X-ray treatment revealed declines in p56/p53^{lyn} signals (FIG. 10A).

Similar findings were obtained when using enolase as the substrate. While stimulation of p56/p53^{lyn} autophosphorylation was less apparent under these conditions, increases in enolase phosphorylation were clearly detectable when comparing anti-Lyn immunoprecipitates from control and irradiated HL-60 cells (FIG. 10B). This increase in activity was rapid and sustained for at least 12 hours (FIG. 10B). Quantitation of ³²P-incorporation into enolase by scintillation counting demonstrated X-ray-induced increases in p56/p53^{lyn} activity of approximately 3-fold at 15 min to 12 hours (FIG. 10B). As observed in autophosphorylation studies, enolase phosphorylation was also decreased at 24 hours (FIG. 10B).

Similar studies were performed at different doses of ionizing radiation (FIG. 11). Treatment with 25 cGy had little if any effect on phosphorylation of p56/p53^{lyn} or enolase. Doses of 50 cGy, however, were associated with increases in p56/p53^{lyn} activity (FIG. 11). Moreover, on the basis of enolase phosphorylation there was an apparent dose-dependent stimulation of this kinase (FIG. 11).

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The cellular effects of ionizing radiation are believed to be related to direct interaction of X-rays with DNA or through the formation of reactive oxygen intermediates (ROIs) which damage DNA and cell membranes (Hall, 1988). While the role of different classes of ROIs in activation of the Src-like kinases is unclear, recent studies have demonstrated that H_2O_2 and diamide, which oxidize free
5 sulfhydryl groups in cells, activate $p56^{lck}$ in T cells (Nakamura *et al.*, 1993).

HL-60 cells were either treated with H_2O_2 for the indicated times or pretreated with 30 mM NAC for 1 hour, irradiated (200 cGy) and harvested at 12
10 hours. Irradiated HL-60 cells treated with H_2O_2 did not show a detectable increase in phosphorylation of $p56/p53^{lyn}$ or enolase (FIG. 12A). Cells were also treated with the antioxidant NAC (Roederer *et al.*, 1990; Staal *et al.*, 1990), an agent that abrogates oxidative stress by scavenging certain ROIs and increasing intracellular glutathione levels (Aruoma *et al.*, 1989; Burgunder *et al.*, 1989). NAC had little
15 effect on X-ray-induced $p56/p53^{lyn}$ activity (FIG. 12A), while this agent completely blocks induction of c-jun and EGR-1 gene expression in irradiated HL-60 cells (Datta *et al.*, 1992b; 1993).

HL-60 cells were treated with 10 μ M herbimycin (H) or 10 μ M genistein
20 (G) for 1 hour, irradiated (200 cGy) and then harvested at 12 hours. Cell lysates were immunoprecipitated with anti-Lyn and the immunoprecipitates were analyzed for phosphorylation of $p56/p53^{lyn}$ and enolase. In marked contrast, the tyrosine kinase inhibitors, herbimycin and genistein inhibited X-ray-induced $p56/p53^{lyn}$
25 activity (FIG. 12B).

Previous work has demonstrated that both ionizing radiation and H_2O_2 are potent inducers of c-jun gene transcription (Datta *et al.*, 1992b). These two agents have also been used to support the role of ROIs in targeting CC(A/T)₆GG sequences to mediate activation of the EGR-1 gene (Datta *et al.*, 1993). The
30 finding that such induction of early response gene transcription is inhibited by NAC further supports the role of some of these intermediates in X-ray-induced nuclear signaling mechanisms.

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The present invention provides for the activation of p56/p53^{lyn} as a distinct cellular response to ionizing radiation and not to H₂O₂-induced oxidative stress.

These findings contrast work by others which suggested that Src-like tyrosine kinases, including p56/p53^{lyn}, are not responsible for signaling in irradiated B cells (Uckun *et al.*, 1992a). The demonstration that ionizing radiation, and not H₂O₂, induces p56/p53^{lyn} activity by an NAC-insensitive mechanism therefore indicates that activation of this tyrosine kinase is independent from those signals responsible for X-ray-induced early response gene expression.

- 10 The finding in B cells that p56/p53^{lyn} is functionally associated with the cell surface (Yamanashi *et al.*, 1992) suggests that activation of this kinase by ionizing radiation may be generated near the plasma membrane rather than in the nucleus. Indeed, the available evidence supports the involvement of receptor-mediated signaling in the activation of p56/p53^{lyn} (Yamanashi *et al.*, 1992; Pleiman *et al.*, 1993). Src-like proteins may be activated through dephosphorylation by tyrosine phosphatases (Mustalin & Altman, 1990; Cantley *et al.*, 1991; Hartwell & Weinart, 1989) and potentially other mechanisms (Cantley *et al.*, 1991; Hartwell & Weinart, 1989).

- 20 In regard to the effect of ionizing radiation on the phosphorylation of p34^{cdc2} on tyrosine, HL-60 cells were grown in RPMI 1640 medium containing 15% heat-inactivated total bovine serum supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 2mM L-glutamine. Exponentially growing cells were suspended in serum free media 18 h prior to irradiation. Irradiation was performed at room temperature using a Gammacell 1000 (Atomic Energy of Canada, Ottawa) with a ¹³⁷Cs source emitting at a fixed dose rate of 13.3 Gy/min as determined by dosimetry.

- 30 Cells were washed twice with ice cold phosphate buffered saline and lysed in buffer A (10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 50 mM NaCl, 5 mM β-glycerophosphate, 1% Triton X-100, 0.5% NP-40, 1 mM sodium vanadate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 10 µg/ml of leupeptin and

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aprotinin). Insoluble material was removed by centrifugation at 14000 rpm for 5 min at 4°C. Protein concentration was determined by Coomassie Blue staining using BSA as standard.

- 5 Soluble proteins (50 µg) were separated by electrophoresis in 10% SDS--polyacrylamide gels and then transferred to nitrocellulose paper. The residual binding sites were blocked by incubating the filter in 5% dry milk in PBST (PBS/0.05% Tween 20) for 1 h at room temperature. The filters were then incubated for 1 h with either mouse anti-phosphotyrosine (anti-P-Tyr; 4G10)
- 10 monoclonal antibody (4G10, UBI, Lake Placid, NY) or a mouse anti-p34^{cdc2} monoclonal antibody which is unreactive with other cyclin-dependent kinases (sc-54; Santa Cruz Biotechnology, Santa Cruz, CA). After washing twice with PBST, the blots were incubated with anti-mouse or anti-rabbit IgG peroxidase conjugate (Sigma Chemical Co., St. Louis, Mo). The antigen-antibody complexes were
- 15 visualized by chemiluminescence (ECL detection system, Amersham, Arlington Heights, IL).

- Immunoprecipitations were performed with anti-P-Tyr or anti-p34^{cdc2} at 5 µg/ml cell lysate. Immune complexes were collected with protein A-Sepharose
- 20 (Pharmacia) and immunoprecipitates were analyzed by 10% SDS-PAGE. After transfer to nitrocellulose and blocking, immunoblot analysis was performed with either anti-p34^{cdc2} or anti-P-Tyr and detected with the appropriate HRP-conjugated second antibody using the ECL system.

- 25 HL-60 cells were exposed to 200 cGy ionizing radiation and monitored for proteins with increased levels of phosphotyrosine. Using an anti-P-Tyr antibody in immunoblot analyses, reactivity with a protein of approximately 34 kD was increased at 1 min after ionizing radiation treatment (FIG. 13A). Similar findings were obtained at 5 and 10 min, while reactivity was decreased at 15 min
- 30 (FIG. 13A). The filters were washed and reprobed with an anti-p34^{cdc2} antibody. The anti-P-Tyr and anti-p34^{cdc2} signals were superimposable. Moreover, there was little detectable change in p34^{cdc2} protein levels following exposure to ionizing

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radiation (FIG. 13B). Similar findings were obtained with doses of ionizing radiation from 50 to 500 cGy (FIG. 14A). The finding that the signals obtained with the anti-p34^{cdc2} antibody (FIG. 14B) were also superimposable over those found with anti-P-Tyr suggested that p34^{cdc2} may undergo phosphorylation on tyrosine following ionizing radiation treatment.

Extracts of irradiated cells were subjected to immunoprecipitation with anti-p34^{cdc2}. The immunoprecipitates were then monitored by immunoblotting with anti-P-Tyr. The signal for p34^{cdc2} was increased in irradiated as compared to control cells (FIG. 15A). While this result further supported increased tyrosine phosphorylation of p34^{cdc2}, the filter was washed and reprobbed with anti-p34^{cdc2} to assay for levels of p34^{cdc2} protein. The finding that the anti-p34^{cdc2} signals were similar in control and irradiated cells (FIG. 15B) indicated that p34^{cdc2} undergoes increased phosphorylation on tyrosine following ionizing radiation exposure.

Activation of p34^{cdc2} requires association with cyclin B (Pines & Hunter, 1989; Russel & Nurse, 1987) and certain posttranslational modifications. In *Schizosaccharomyces pombe*, the p34^{cdc2}/cyclin B complex is inactivated by phosphorylation of p34^{cdc2} on tyrosine 15 by Wee1 (Featherstone & Russell, 1991; Parker *et al.*, 1991; 1992; Gould & Nurse, 1989). Dephosphorylation of p34^{cdc2} on Tyr-15 by the cdc25 gene product is necessary for activation of p34^{cdc2} and entry into mitosis (Gould *et al.*, 1989; Enoch & Nurse, 1990). The wee1 and cdc25 gene products thus determine the timing of entry into mitosis by a series of phosphorylations and dephosphorylations of p34^{cdc2}. Other work in *S. pombe* has demonstrated that mitotic checkpoints monitor DNA synthesis and the presence of DNA damage (Al-Khodairy & Carr, 1992; Rowley *et al.*, 1992; Lock & Ross, 1990). The DNA damage checkpoint evidently regulates p34^{cdc2} by mechanisms distinct from those induced by the replication checkpoint (Rowley *et al.*, 1992; Lock & Ross, 1990). Other studies have demonstrated that p34^{cdc2} kinase activity is decreased when CHO cells are exposed to 8 Gy ionizing radiation (Uckun *et al.*, 1992b).

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The present invention discloses activation of Src-like tyrosine kinases and phosphorylation of tyrosine kinase substrates, such as p34^{cdc2}, as a rapid response to ionizing radiation. Inhibition of the radiation-induced activation of those tyrosine kinases prevents or inhibits substrate phosphorylation. Because the function of those substrates depends on their state of phosphorylation, inhibition of phosphorylation alters the function of those substrates. To the extent that substrate function is responsible for all or part of the cascade of changes associated with radiation, altering substrate function by inhibition of phosphorylation alters the cells response to radiation. Thus, the present invention contemplates a process to alter the response of cell to radiation, the process comprising inhibiting tyrosine kinase activity. In a preferred embodiment, the tyrosine kinase is a Src-like tyrosine kinase of the lyn family.

EXAMPLE III

Activation of c-Abl by DNA Damaging Agents

Human U-927 cells were exposed to 2 Gy IR and harvested at 1 h. U-937 and NIH3T3 cells were treated with 2 Gy IR at room temperature for the indicated times using a Gammacell 1000 (Atomic Energy of Canada, Ottawa) under aerobic conditions with a ¹³⁷Cs source emitting at a fixed dose rate of 0.76 Gy/min as determined by dosimetry. The cells were also treated with 10 μM mitomycin C (MMC, Sigma Chemical Co., St. Louis, MO) for 1 h or 10 μM cisplatinum (CDDP) for 30 min. The cells were swelled in 2 ml of ice cold hypotonic lysis buffer [1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 2 mM MgCl₂, 10 mM KCl, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 10 μg/ml each of pepstatin, leupeptin and aprotinin] for 30 min and then subjected to Dounce homogenization (15-25 strokes, tight pestle A).

The resulting lysate was loaded onto 1.5 ml of buffer A [1 M sucrose in hypotonic lysis buffer containing the protease and phosphatase inhibitors] and centrifuged at 1600g for 15 min to pellet nuclei. The pellet was washed and solubilized in buffer A containing 1% NP-40. Anti-c-Abl immunoprecipitations

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were performed by adding the K12 anti-Abl antibody and Protein A-Sepharose for 2h at 4°C.

5 Nuclear lysates were subjected to immunoprecipitation with anti-abl antibody and the immunoprecipitates were assayed for phosphorylation of a GST-Crk fusion protein. Immune complex kinase assays were performed by incubating the resulting protein complexes in kinase buffer [50 mM Tris, pH 7.5, 10 mM MnCl_2 , 1 mM DTT], with either 5 μg GST-Crk(120-225) or GST-Crk(120-212), 2-5 μCi [γ - ^{32}P]ATP (New England Nuclear, Boston, MA) for 30 min at 28°C
10 and analyzed by 10% SDS-PAGE and autoradiography.

In the peptide phosphorylation assays, immune complexes were incubated in kinase buffer with 20 μM peptide [EAIYAAPFAKKK; SEQ ID NO:5], 10 μM ATP and 2-5 μCi [γ - ^{32}P]ATP for 4 min at 25°C. After incubation, 25 μl was
15 spotted onto phosphocellulose discs, followed by washing with 1% phosphoric acid and then distilled water. The incorporated [^{32}P] phosphate was determined by scintillation counting.

The c-Crk protein contains an N-terminal SH2 domain followed by two
20 SH3 domains. c-Abl binds to the N-terminal SH3 domain of Crk and phosphorylates Tyr221 (Feller *et al.*, 1994; Ren *et al.*, 1994). There was a low level of GST-Crk(120-225) phosphorylation with anti-Abl immunoprecipitates from control cells, while IR exposure was associated with stimulation (4-5-fold) of Crk kinase activity (FIG. 16A).

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In contrast, there was no detectable IR-induced phosphorylation of a GST-Crk(120-212) fusion protein which lacks Tyr221 (FIG. 16C). The finding that IR has no detectable effect on c-Abl levels supported an increase in c-Abl activity (FIG. 16A).

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NIH3T3 fibroblasts were used in similar studies to determine whether activation of c-Abl is detectable in different cell types. The results demonstrate

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that IR treatment is also associated with stimulation of c-Abl activity in these cells (FIG. 16B). In order to further assess activation of c-Abl by IR treatment, the inventors used a peptide (EAIYAAPFAKKK; SEQ ID NO:5) recently identified as a specific substrate for c-Abl activity (Songyang *et al.*, 1995). Anti-Abl immunoprecipitates from irradiated U-937 cells exhibited maximal (nearly 5-fold) phosphorylation of the peptide substrate at 1 h (FIG. 16D). In contrast, immunoprecipitates prepared with preimmune rabbit serum failed to exhibit IR-induced phosphorylation of the peptide (FIG. 16D).

Since IR induces single and double DNA strand breaks, the inventors asked whether treatment with other agents that damage DNA is also associated with c-Abl activation. Cisplatin (CDDP) forms DNA intrastrand crosslinks (Sherman and Lippard, 1987), while mitomycin C (MMC) forms monofunctional and bifunctional DNA lesions (Tomasz *et al.*, 1988). Treatment of NIH3T3 cells with these alkylating agents was associated with an increase in c-Abl activity which was similar to that obtained following IR exposure (FIG. 16E, FIG. 16F). These findings suggest that c-Abl is activated by diverse DNA damaging agents.

EXAMPLE IV

Involvement of SAP Kinases in Response to DNA Damaging Agents

Two serines (Ser63 and Ser73) in the trans-activation domain of c-Jun have been identified as substrates for the stress-activated protein (SAP) kinases (Pulverer *et al.*, 1991; Kyriakis *et al.*, 1994; Derijard *et al.*, 1994). The SAP kinases are activated in response to treatment with TNF, anisomycin and UV lights. The finding that Ha-Ras is involved in the stimulation of SAP kinase by UV light has suggested that this signaling cascade is initiated by damage to cellular components other than DNA (Derijard *et al.*, 1994; Engelberg *et al.*, 1994). There is presently little known about the involvement of SAP kinase in the cellular response to DNA-damaging agents.

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To address this issue, the inventors analyzed anti-SAP kinase immunoprecipitates for phosphorylation of the trans-activation domain of c-Jun. GST-Jun(2-100) fusion protein was prepared as described (Salem *et al.*, 1995). The Abl-/- fibroblast cell line was obtained from David Baltimore's laboratory.

Using GST-Jun(2-100) as a substrate, there was a low level of Jun phosphorylation with anti-SAP kinase immunoprecipitates from NIH3T3 cells and this activity was increased by IR exposure (FIG. 17A). Similar findings were obtained with immunoprecipitates from CDDP- or MMC-treated cells (FIG. 17A).

Since the above findings supported activation of SAP kinase by DNA-damaging agents, the inventors asked whether c-Abl is involved in a cascade that includes SAP kinase. Mouse fibroblasts deficient in c-Abl (Abl-/-, derived from mice with targeted c-Abl disruption) (Tybulewicz *et al.*, 1991) were assayed for SAP kinase activity following irradiation or drug treatment. In the Abl-/- cells, there was no detectable induction of SAP kinase activity after exposure to IR, CDDP or MMC (FIG. 17B), while immunoblot analysis with anti-SAP kinase demonstrated expression of this protein before and after treatment of these cells. These findings suggested that c-Abl is necessary for activation of SAP kinase in cells treated with DNA-damaging agents.

EXAMPLE V

c-Abl Mediates Signals in the SAP Kinase Stress Response Pathway

In order to more definitively demonstrate a role for c-Abl in a cascade that contributes to activation of SAP kinase, the inventors stably expressed c-Abl in the Abl-/- cells (designated Abl+). Abl-/- cells were reconstituted with the c-Abl gene by retroviral transduction. The c-Abl (murine type IV) gene was subcloned into the pBaBe-puro retroviral expression vector (Morgenstern and Land, 1990). Helper-free retrovirus was generated and used to infect Abl-/- cells as described

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(Pear *et al.*, 1993). Puromycin selected cells were used in experiments following removal from drug for 18 h.

5 The level of c-Abl expression in the Abl+ cells was readily detectable, but somewhat lower than that in NIH3T3 cells (FIG. 18A). IR treatment of the Abl+ cells was associated with stimulation of c-Abl activity (FIG. 18B). More importantly, exposure of the Abl+ cells to IR was associated with increases in SAP kinase activity (FIG. 18C). MMC treatment of the Abl+ cells also resulted in activation of both c-Abl and SAP kinase activities (FIG. 18C). Taken together, 10 these results provide definitive evidence that c-Abl mediates signals in the SAP kinase stress response pathway.

EXAMPLE VI

c-Abl is Not Involved With TNF- α Induced Stress

15 The finding that DNA-damaging agents activate c-Abl suggested that this event may be a generalized response to cellular stress. Previous work has shown that the stress pathway involving SAP kinase is activated by TNF (Kyriakis *et al.*, 1994). However, TNF had little if any effect on c-Abl activity in NIH3T3 cells 20 (FIG. 19A). In contrast, TNF stimulated SAP kinase activity in these cells. Moreover, TNF induced SAP kinase activity in the Abl-/- cells (FIG. 19B). These findings suggest that c-Abl is selectively activated by agents (IR, CDDP, MMC) that act primarily by damaging DNA, while c-Abl is not involved in the response to TNF-induced cellular stress.

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EXAMPLE VII

DNA Damaging Agents

30 Following radiation exposure, many single strand breaks are produced in DNA, but these are readily repaired using the opposite strand of DNA as a template. X-ray energy deposition on DNA may lead not only to strand breakage but to base damage. The breakage may result in incorrect rejoining in pre-

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replication chromosomes in the G_1 phase, leading to chromosomal aberrations, or if the radiation is given late in S or G_2 , chromatid aberrations will result.

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

A variety of other DNA damaging agents may be used with the tyrosine kinase inhibitors, as provided by this invention. This includes agents that directly crosslink DNA, agents that intercalate into DNA, and agents that lead to chromosomal and mitotic aberrations by affecting nucleic acid synthesis.

Agents that induce DNA alkylation, such as mitomycin C, may be used. Mitomycin C is an extremely toxic antitumor antibiotic that is cell cycle phase-nonspecific. It is almost always given intravenously, at a dose of 20 mg/m^2 , either in a single dose or given in 10 separate doses of 2 mg/m^2 each given over 12 days. It has been used clinically against a variety of adenocarcinomas (stomach, pancreas, colon, breast) as well as certain head and neck tumors.

Another option is to employ cisplatin, which has also been widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m^2 for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intratumorally or intraperitoneally.

Agents that damage DNA also include compounds that interfere with DNA replication, mitosis, and chromosomal segregation. Examples of these compounds include adriamycin, also known as doxorubicin, etoposide, verapamil,

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podophyllotoxin, and the like. Widely used in clinical setting for the treatment of neoplasms these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m² at 21 day intervals for adriamycin, to 35-50 mg/m² for etoposide, intravenously or double the intravenous dose orally.

5

Agents that disrupt the synthesis and fidelity of nucleic acid precursors, and subunits also lead to DNA damage. As such a number of nucleic acid precursors have been developed. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5-fluorouracil (5-FU), are preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

10

Therefore, the DNA damaging agents or factors defined herein include any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include ionizing radiation and waves that induce DNA damage, such as, γ -irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as "chemotherapeutic agents", function to induce DNA damage, all of which are intended to be of use in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated to be of use, include, e.g., alkylating agents such as mitomycin C, adozelesin, cis-platinum, and nitrogen mustard. The invention also encompasses the use of a combination of one or more DNA damaging agents, whether ionizing radiation-based or actual compounds, with one or more tyrosine kinase inhibitors.

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EXAMPLE VIII

Tyrosine Kinase Inhibitors

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Tyrosine protein kinase activities are known to be associated with oncogene products of the retroviral src gene family, and also with several cellular growth

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factor receptors such as that for epidermal growth factor (EGF). Activation of protein tyrosine phosphorylation by p56/p53^{lyn} in the present studies demonstrates that the lyn protein is associated with the cell cycle regulatory protein p34^{cdc2}, contributing to mitotic arrest. If this association is blocked, such as by use of protein tyrosine kinase inhibitors such as genistein or herbimycin A, the cells are unable to arrest in the G₂ phase, forcing cell cycle traverse and expression of potentially lethal damage. Thus, the combined use of DNA damaging agents such as ionizing radiation or alkylating agents with tyrosine kinase inhibitors is a novel approach to enhancing cell killing.

Genistein, a natural isoflavonoid phytoestrogen, has been reported to exhibit specific inhibitory activity against tyrosine kinases of EGF receptor, pp60^{v-src} and pp110^{gag-fes}. It has been generally shown to block a number of EGF dependent phenomena, including both receptor autophosphorylation and histone phosphorylation.

Herbimycin A has also been shown to inhibit the autophosphorylation of EGF-stimulated receptors in intact cells in a time and dose dependent manner. Herbimycin A both decreases the receptor quantity and the EGF-stimulated receptor kinase activity.

Other tyrosine kinase inhibitors may also be used, for example, those isolated from natural sources. One such compound is erbstatin (Umezawa and Imoto M, 1991; Sugata *et al.*, 1993) and its analogues, e.g., RG 14921 (Hsu *et al.*, 1992). Lavendustin A from *Streptomyces griseolavendus* (Onoda *et al.*, 1989), which is about 50 times more inhibitory than erbstatin, and analogues thereof, are also contemplated for use as protein-tyrosine kinase inhibitors (Smyth *et al.*, 1993b). Picetannol (3,4,3',5'-tetrahydroxy-trans-stilbene; Geahlen and McLaughlin, 1989) and polyhydroxylated stilbene analogues thereof (Thakkar *et al.*, 1993) may also be used.

Further natural tyrosine kinase inhibitors that may be used are emodin (3-methyl-1,6,8-trihydroxyanthraquinone), an inhibitor from the Chinese medicinal

- plant *Polygonum cuspidatum* (Jayasuriya *et al.*, 1992; Chan *et al.*, 1993); desmal (8-formyl-2,5,7-trihydroxy-6-methylflavanone), isolated from the plant *Desmos chinensis* (Kakeya *et al.*, 1993); the chlorosulfolipid, malhamensilpin A, isolated from the cultured chrysophyte *Poterioochromonas malhamensis* (Chen *et al.*, 1994); flavonoids obtained from *Koelreuteria henryi* (Abou-Shoer *et al.*, 1991); fetuin, a natural tyrosine kinase inhibitor of the insulin receptor (Rauth *et al.*, 1992).

- Another group of compounds known to be tyrosine kinase inhibitors are the tyrphostins, which are low molecular weight synthetic inhibitors (Gazit *et al.*, 1989). The tyrphostins AG17, AG18, T23 and T47 have been shown to inhibit pancreatic cancer cell growth *in vitro* (Gillespie *et al.*, 1993). Tyrphostins have also been shown to have antiproliferative effects on human squamous cell carcinoma *in vitro* and *in vivo* (Yoneda *et al.*, 1991). RG-13022 and RG-14620 were found to suppress cancer cell proliferation *in vitro* and tumor growth in nude mice. Another active tyrphostin is AG879 (Ohmichi *et al.*, 1993).

- Various chemical compounds may also be used in combination with DNA damaging agents, such as ionizing radiation, as have been described in the literature for use alone. One example is RG50864 (Merkel *et al.*, 1993). Further examples are the indole substituted 2,2'-dithiobis(1-methyl-N-phenyl-1H-indole-3-carboxamides, especially the 5-substituted derivative, as described by Rewcastle *et al.* (1994). (Z)-alpha-[(3,5-dichlorophenyl)methylene]-3-pyridylacetone (RG 14620) is another active tyrosine kinase inhibitor that may be used in a topical or intravenous form (Khetarpal *et al.*, 1994).

- BE-23372M, (E)-3-(3,4-dihydroxybenzylidene)-5-(3,4-dihydroxyphenyl)-2(3H)-furanone, is also a tyrosine kinase inhibitor (Tanaka *et al.*, 1994a). This may be synthesized from 3-(3,4-dimethoxybenzoyl)propionic acid and veratraldehyde or 3,4-diacetoxy-benzaldehyde, as described by Tanaka *et al.* (1994b). BE-23372M may also be isolated from the culture broth of a *Rhizoctonia*

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solani fungus (strain F23372) using acetone and then purified by solvent extraction and column chromatography (Okabe *et al.*, 1994).

Further tyrosine kinase inhibitors that may be used include

- 5 4,5-Dianilinophthalimide, which has, alone, been shown to have *in vivo* antitumor activity (Buchdunger *et al.*, 1994). Hydroxylated 2-(5'-salicyl)naphthalenes form another group of inhibitors that could be used in the present invention, and may be prepared as described by Smyth *et al.* (1993a).

- 10 To kill a cell in accordance with the present invention, one would generally contact the cell with a DNA damaging agent and a tyrosine kinase inhibitor in a combined amount effective to kill the cell. The term "in a combined amount effective to kill the cell" means that the amount of the DNA damaging agent and inhibitor are sufficient so that, when combined within the cell, cell death is
- 15 induced. Although not required in all embodiments, the combined effective amount of the two agents will preferably be an amount that induces more cell death than the use of either element alone, and even one that induces synergistic cell death in comparison to the effects observed using either agent alone. A number of *in vitro* parameters may be used to determine the effect produced by the compositions and
- 20 methods of the present invention. These parameters include, for example, the observation of net cell numbers before and after exposure to the compositions described herein.

- Similarly, a "therapeutically effective amount" is an amount of a DNA
- 25 damaging agent and tyrosine kinase inhibitor that, when administered to an animal in combination, is effective to kill cells within the animal. This is particularly evidenced by the killing of cancer cells within an animal or human subject that has a tumor. "Therapeutically effective combinations" are thus generally combined amounts of DNA damaging agents and tyrosine kinase inhibitors that function to
- 30 kill more cells than either element alone and that reduce the tumor burden.

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EXAMPLE IX

Antisense Constructs

5 The present invention generally relates to methods of inhibiting or down-regulating the expression of the c-Abl gene through the preparation and use of antisense constructs that are complementary to distinct regions of the c-Abl gene. The nucleotide sequences of the c-Abl gene are set forth in SEQ ID NO: 1 and SEQ ID NO: 2.

10 Generally speaking, to practice the present invention, one would use representative methods for cloning the c-Abl gene, as described in the literature (Shtivelman *et al.*, 1986). This publication should provide adequate direction where one seeks to obtain a c-Abl sequence.

15 A preferred method for cloning c-Abl sequences is through the application of PCR-amplified cloning. In this relatively well known technique, one employs oligonucleotide primers complementary to c-Abl, as may be determined from the sequences set forth in SEQ ID NO: 1 and SEQ ID NO: 2, that allow the specific amplification of the c-Abl gene sequence.

20 Recombinant clones that incorporate c-Abl DNA are readily achieved through the PCR amplification of the distinct coding region using primers incorporating the amplified DNA into a recombinant clone, and selecting recombinant clones that have received the c-Abl DNA bearing clones. One
25 generally clones restriction digested fragments of the desired gene into an appropriate plasmid that is amplified when grown in an appropriate prokaryotic host cell. Following amplification, the c-Abl DNA containing clones are then purified, and preferably, the cloned DNA is sequenced sufficiently to ensure that it contains the desired sequences.

30 c-Abl DNA is then removed from the vector employed for c-Abl DNA cloning, and used in the construction of appropriate antisense vectors. This will

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entail, of course, placing the c-Abl DNA in an antisense direction behind an appropriate promoter and positioned so as to bring the expression of the antisense c-Abl under control of the promoter.

5 When selecting primers for c-Abl amplification, one typically desires to use primers such that at least about 40-50 and preferably about 100-200 nucleotides of the c-Abl gene are amplified and thereby cloned. It is generally believed that the larger the region of c-Abl gene sequence that is cloned, the better the down-regulation of the targeted gene.

10

 The particular vector used for introduction of antisense c-Abl coding sequences is not believed to be particularly crucial to the practice of the present invention, so long as the vector is capable of introducing the nucleic acid coding sequences into the genome of the targeted cell in a relatively stable manner. By
15 way of illustration, preferred vectors may be a retrovirus, adenovirus, or HSV-1.

EXAMPLE IX

Treatment Protocols

20 **Treatment with Tyrosine Kinase Inhibitors**

- 1) Patients exhibiting neoplastic disease are treated with a protein kinase inhibitor, for example genistein, at a concentration of between
1 and 100 μ M, or herbimycin A at a concentration of between about
25 1 and 100 μ M, for 6 hours prior to exposure to a DNA damaging agent.
- 2) Patients are exposed to ionizing radiation (2 gy/day for up to 35 days), or an approximate a total dosage of 700 gy.

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- 3) As an alternative to ionizing radiation exposure, patients are treated with a single intravenous dose of mitomycin C at a dose of 20 mg/m².

5 It is contemplated that mitomycin C treatment in combination with tyrosine protein kinase inhibitors will be effective against cancer of the stomach, pancreas, oral cavity, breast and head/neck.

Treatment with Antisense Sequences

10

- 1) Patients exhibiting neoplastic disease are treated with an antisense RNA molecule that comprises a sequence that is complementary to a region of the c-Abl gene and hybridizes to such a region. This antisense RNA molecule may be in combination with a recombinant vector that comprises a nucleic acid sequence capable of expressing the antisense RNA in the cell. The vector is introduced into the cell in a manner that allows expression of the encoded antisense RNA at a level sufficient to inhibit gene expression.

15

- 20 2) Patients are exposed to ionizing radiation (2 Gy/day for up to 35 days), or an approximate a total dosage of 700 Gy.
- 3) As an alternative to ionizing radiation exposure, patients are treated with a single intravenous dose of mitomycin C at a dose of 20 mg/m².

25

* * *

30 All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in

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the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related
5 may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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SEQUENCE LISTING

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5

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(F) POSTAL CODE (ZIP): 60637

10

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(B) STREET: 44 Binney Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: United States of America
(F) POSTAL CODE (ZIP): 02115

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- (ii) TITLE OF INVENTION: METHODS AND COMPOSITIONS
INCLUDING DNA DAMAGING
AGENTS AND TYROSINE KINASE
INHIBITORS OR ACTIVATORS

25

- (iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version
#1.30 (EPO)

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(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/520,923
(B) FILING DATE: 30-AUG-1995

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3780 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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15 GAGGGGTTA AGGCGCAGGC GCGCGCGGGG CCGGGCGGGC CCTGCGGGC GCCCTCTCCG 180

GGCCCTTTGT TAACAGCGCG GTCCCGGCCA GCGGAGACGC GCGCGCCCTG GCGGGGCGCG 240

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GCCTGAGCCG GGGCCGGACC GAGCTGGAG AGGGGTCCG GCCCGATCGT TCGCTTGGCG 360

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-72-

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5520 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

5 CGCGCCGCC CTGGGCGGGC GCGGGCGGCG GCGGCGGGTG AGGCGGCGCT GCGGGCGGCG 60
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-79-

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-81-

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-83-

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10

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ile Glu Lys Ile Gly Glu Gly Thr Tyr Gly Val Val Tyr Lys
 1 5 10

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

-85-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ile Glu Lys Ile Gly Glu Gly Thr Phe Gly Val Val Tyr Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Glu Ala Ile Tyr Ala Ala Pro Phe Ala Lys Lys Lys
1 5 10

- 86 -

CLAIMS:

1. An antisense RNA molecule that inhibits the expression of a gene product of c-Abl, the antisense molecule including a region that is complementary to and
5 that hybridizes with a region of the selected gene.
2. The antisense molecule according to claim 1, wherein the antisense RNA molecule is capable of selectively inhibiting the expression of the c-Abl gene
10 product over that of another member of the non-receptor type of tyrosine kinase.
3. The antisense molecule according to claim 1, wherein the RNA molecule comprises a sequence that is complementary to an exon region sequence of the c-
15 Abl gene.
4. A DNA molecule that expresses an antisense RNA molecule in accordance with claim 2.
20
5. A nucleic acid molecule comprising a coding region that expresses an antisense RNA molecule that selectively inhibits the gene product of the c-Abl gene, the DNA coding region including an antisense RNA coding region that is
25 complementary to a region of the c-Abl gene.
6. The nucleic acid molecule according to claim 5, further defined as a DNA molecule.
30

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7. The nucleic acid molecule according to claim 6, wherein the DNA molecule encodes a RNA molecule having a sequence that is complementary to the c-Abl gene sequence.

5

8. The nucleic acid molecule according to claim 6, wherein the DNA encodes a RNA molecule having a sequence that is complementary to a 2000 base region of the c-Abl gene.

10

9. The nucleic acid molecule according to claim 6, wherein the DNA encodes a RNA molecule having a sequence that is complementary to a 1000 base region of the c-Abl gene.

15

10. The nucleic acid molecule according to claim 6, wherein the DNA encodes a RNA molecule having a sequence that is complementary to a 500 base region of the c-Abl gene.

20

11. The nucleic acid molecule according to claim 6, wherein the DNA encodes a RNA molecule having a sequence that is complementary to a 100 base region of the c-Abl gene.

25

12. The nucleic acid molecule according to claim 6, wherein the DNA encodes a RNA molecule having a sequence that is complementary to a 10 base region of the c-Abl gene.

30

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13. The nucleic acid molecule according to claim 6, wherein the coding region is positioned under the control of a promoter that is capable of being expressed in a mammalian cell.

5

14. The nucleic acid molecule according to claim 13, wherein the coding region is positioned under the control of a promoter that is capable of being expressed in a human cell.

10

15. An expression vector comprising a gene encoding a RNA molecule complementary to the c-Abl gene and positioned under the control of a promoter, the gene positioned to effect transcription of the c-Abl gene in an orientation opposite to that of vector transcription.

15

16. The vector according to claim 15, wherein the encoded antisense RNA molecule is capable of selectively inhibiting the expression of the c-Abl gene product.

20

17. A pharmaceutical composition comprising the vector according to claim 15.

25

18. A method of selectively inhibiting the expression of c-Abl in a cell comprising

- (a) preparing an antisense RNA molecule that includes a region that is complimentary and capable of hybridizing with a region of the c-Abl gene; and

30

- 89 -

- (b) introducing the antisense RNA molecule into the cell in an amount effective to inhibit the expression of the c-Abl gene.
- 5 19. A method of selectively inhibiting the expression of c-Abl while treating a patient with DNA damaging agents comprising
- 10 (a) administering to the patient a dose of a DNA damaging agent in an amount effective to produce an increase in c-Abl production; and
- (b) administering to the patient an effective amount of an agent that inhibits the expression of the c-Abl gene.
- 15 20. The method according to claim 19, wherein the inhibiting agent is an antisense RNA molecule that includes a region that is complimentary and capable of hybridizing with a region of the c-Abl gene.
- 20 21. The method according to claim 20, wherein the antisense RNA molecule is introduced into the cell by introduction of a DNA molecule that encodes and expresses the antisense RNA molecule.
- 25 22. The method according to claim 21, wherein the DNA molecule is introduced into the cell by a liposome.
- 30 23. The method according to claim 21, wherein the DNA molecule is introduced into the cell by a virus.

- 90 -

24. The method according to claim 23, wherein the virus is an adenovirus.
25. The method according to claim 23, wherein the virus is a retrovirus.
26. The method according to claim 23, wherein the virus is HSV-1.
27. The method according to claim 19, wherein the DNA damaging agent is ionizing radiation.
28. The method according to claim 19, wherein the DNA damaging agent is mitomycin C.
29. A method of selectively inhibiting the expression of c-Abl in a cell, comprising:
- (a) preparing an antisense RNA molecule that comprises a sequence that is complementary to a region of the c-Abl gene and hybridizes to such a region;
 - (b) preparing a recombinant vector that comprises a nucleic acid sequence capable of expressing the antisense RNA in the cell; and
 - (c) introducing the vector into the cell in a manner that allows expression of the encoded antisense RNA at a level sufficient to inhibit gene expression.

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30. In combination, a pharmaceutical composition comprising a DNA damaging agent in combination with an antisense RNA molecule that comprises a sequence that is complementary to a region of the c-Abl gene and hybridizes to such a region.

5

31. The method according to claim 30, wherein the DNA damaging agent is ionizing radiation.

10

32. The method according to claim 30, wherein the DNA damaging agent is mitomycin C.

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FIG. 1A

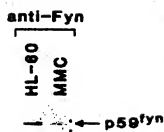


FIG. 1B

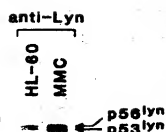


FIG. 1C

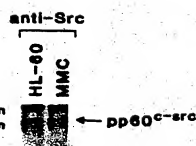


FIG. 1D

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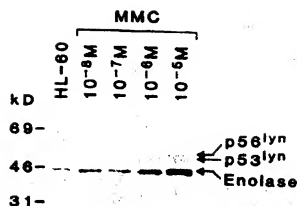


FIG. 2A

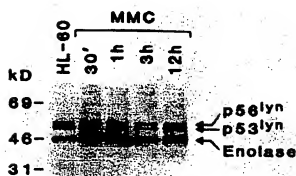


FIG. 2B

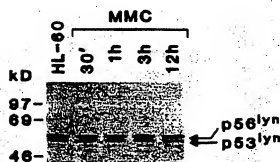


FIG 2C

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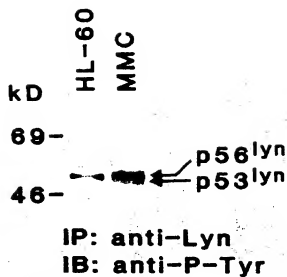


FIG. 3A

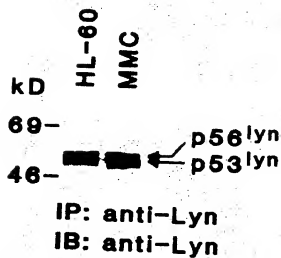


FIG. 3B

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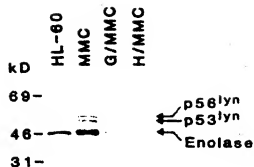


FIG. 4A

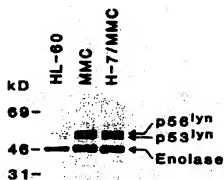


FIG. 4B

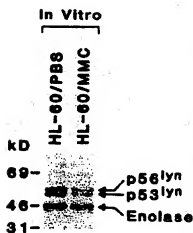


FIG. 4C

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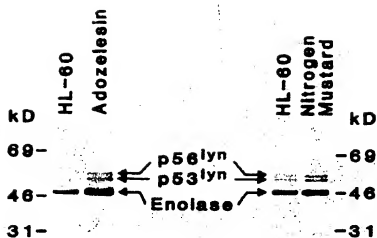


FIG. 5A

FIG. 5B

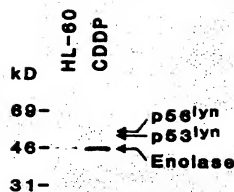


FIG. 5C

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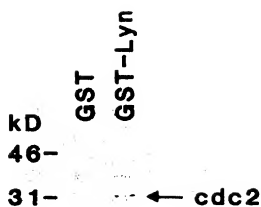


FIG. 6A

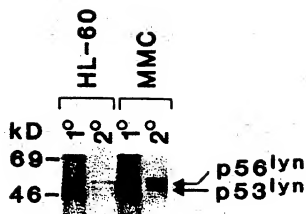
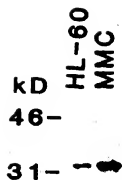


FIG. 6B

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IP: anti-cdc2
IB: anti-P-Tyr

FIG. 7A



IP: anti-cdc2
IB: anti-cdc2

FIG. 7B

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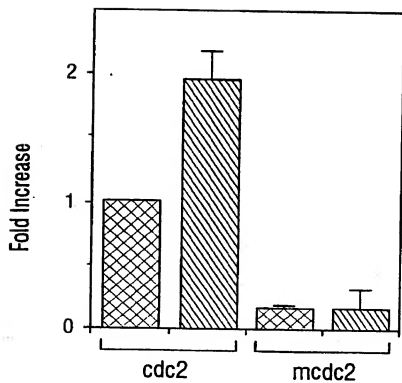


FIG. 8

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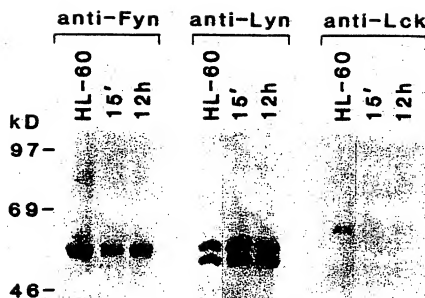


FIG. 9

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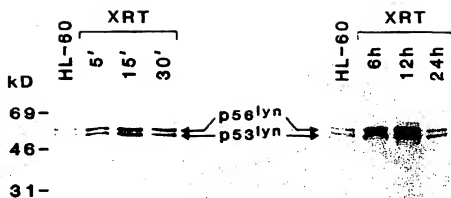


FIG. 10A

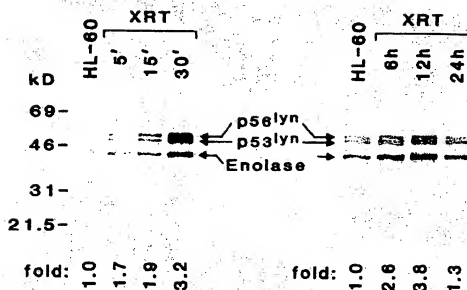


FIG. 10B

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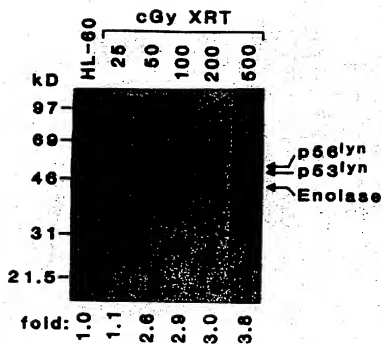


FIG. 11

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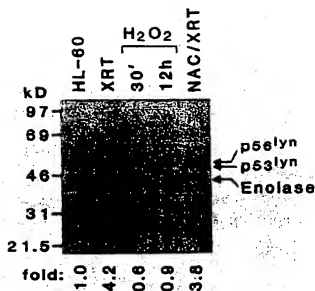


FIG. 12A

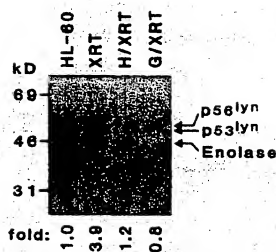


FIG. 12B

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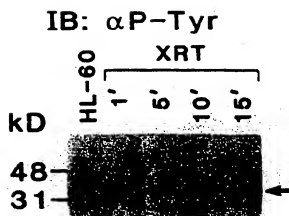


FIG. 13A

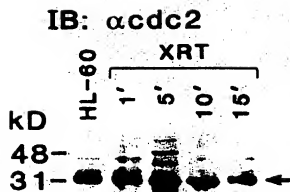


FIG. 13B

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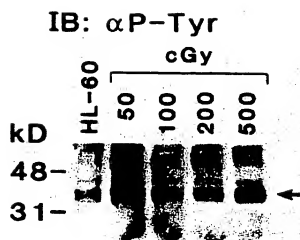


FIG. 14A

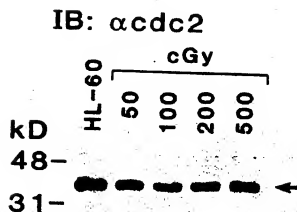


FIG. 14B

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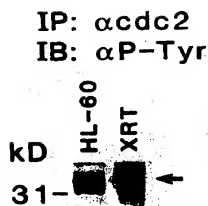


FIG. 15A

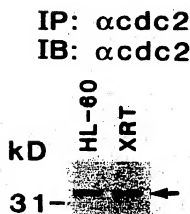


FIG. 15B

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FIG. 16A



FIG. 16B

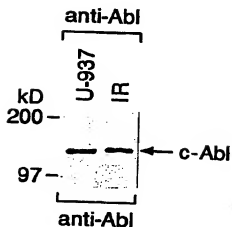


FIG. 16C

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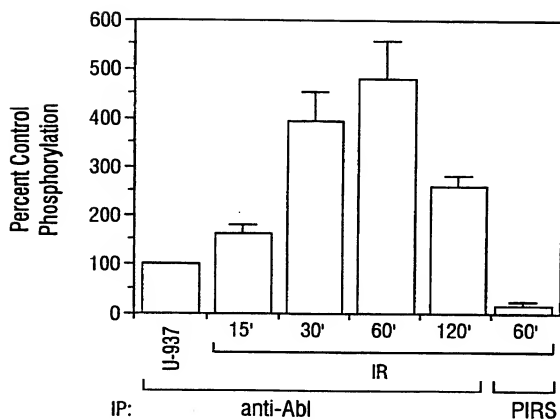


FIG. 16D

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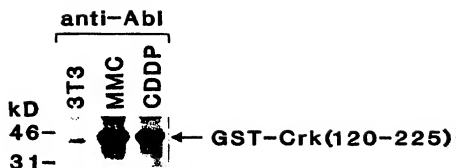


FIG. 16E

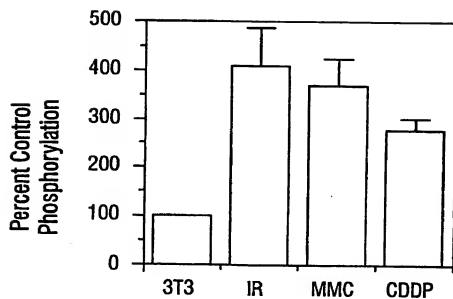


FIG. 16F

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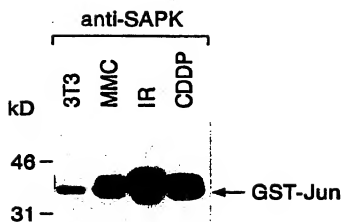


FIG. 19A

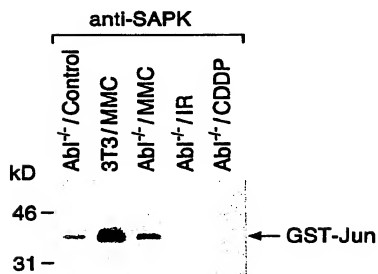


FIG. 19B

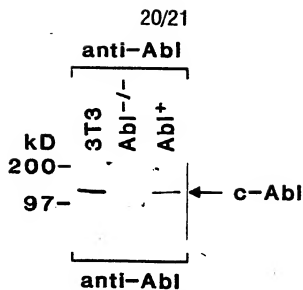


FIG. 18A

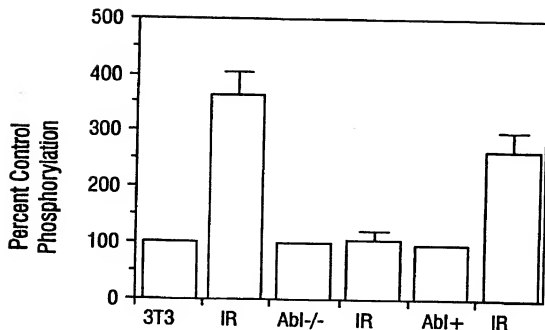


FIG. 18B

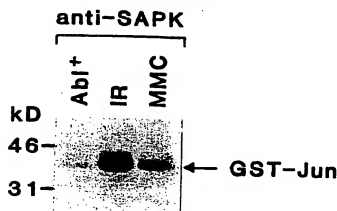


FIG. 18C

SUBSTITUTE SHEET (RULE 26)

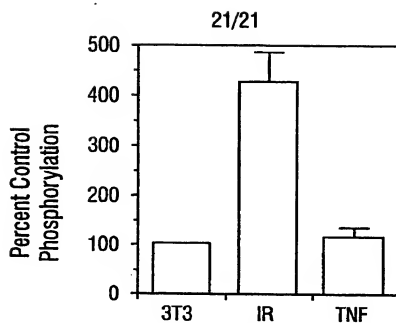


FIG. 19A

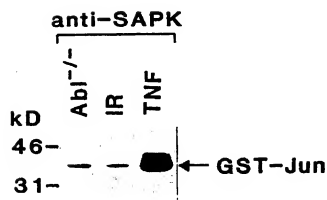


FIG. 19B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13922

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/04; C12N 15/63

US CL : 536/24.5; 435/320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/24.5; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, MEDLINE, EMBASE, BIOSIS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,225,326 A (BRESSER ET AL) 06 July 1993, especially columns 16-17.	1-14
Y	WO 91/03260 A1 (TEMPLE UNIVERSITY OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION) 21 March 1991, see entire document.	1-32
Y	TSENG et al. Antisense oligonucleotide technology in the development of cancer therapeutics. Cancer Gene Therapy. 1994, Vol. 1, No. 1, pages 65-71, see entire document.	1-32
Y	ROSTI et al. c-abl Function in Normal and Chronic Myelogenous Leukemia Hematopoiesis: <i>In Vitro</i> Studies With Antisense Oligomers. Leukemia. January 1992, Vol. 6, No. 1, pages 1-7, see entire document.	1-32

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T
*A document defining the general state of the art which is not considered to be of particular relevance	* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*B document published on or after the international filing date	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O document referring to an oral disclosure, use, exhibition or other means	*Z document member of the same patent family
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

28 OCTOBER 1996

Date of mailing of the international search report

06 NOV 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

D. CURTIS HOGUE, JR.

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1992)*

INTERNATIONAL SEARCH REPORT

Int. application No.
PCT/US96/13922

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CARACCILO et al. Lineage-Specific Requirement of <i>c-abl</i> Function in Normal Hematopoiesis. Science. 08 September 1989, Vol. 245, No. 4922, pages 1107-1110, see entire document.	1-32
Y	ROSTI et al. Oligodeoxynucleotides antisense to <i>c-abl</i> specifically inhibit entry into S phase of CD34-positive hematopoietic cells and their differentiation to granulocyte-macrophage progenitors. Blood. 1994, Vol. 84, No. 10, Suppl. 1, page 125A, abstract no. 487, see entire abstract.	1-32

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